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Epigenetic Pathways Involved in Response to Lead (PB) Exposure in the Aging Brain: Relevance to Alzheimer's Disease

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EPIGENETIC PATHWAYS INVOLVED IN RESPONSE TO LEAD (PB) EXPOSURE IN THE AGING BRAIN: RELEVANCE TO ALZHEIMER'S DISEASE

BY

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ABSTRACT

Alzheimer's Disease (AD) has a complicated pathology with many potential etiologies. In patients, a diagnosis of AD is confirmed at the time of autopsy by the presence of two major pathological hallmarks: the amyloid beta plaque (A**β** plaque) and the neurofibrillary tangle (NFTs). The late-onset (LOAD) form of this disease has no clear genetic etiology, with the exception of an increased risk of carrying the APOE4 allele. Therefore, research has focused on identifying environmental risk factors in its etiology. Our lab has a legacy of studying environmental exposure to lead (Pb) during early development. We have shown in previous publications that exposure to Pb during early life results in the upregulation in a number of key genes in the amyloid and tau pathways directly related to the development of the characteristic hallmarks of AD.

In this dissertation, Manuscript I was a review of the literature related to Pb exposure in both children and adults, and the role Pb has in neurodegenerative diseases, either by workplace exposures or by developmental basis of diseases. The focus of the review was also to present evidence from our work and others to suggest the mechanism by which these changes occur, via potential epigenetic pathways.

Manuscript II was aimed to investigate the involvement of epigenetic pathways in the developmental model of Pb exposure, in which (wild-type mice were exposed to 0.2% Pb from PND 1 and 20 Western blot and RT-PCR analysis were performed for a number of key epigenetic marks and regulators across

the lifespan in the cortex of mice developmentally exposed to Pb. Alterations in a number of key mediators involved in DNA methylation were identified across the lifespan, as well as specific alterations in histone marks involved in both repression (H3K27me3) and activation (H3K9Ac, H3K4me2) pathways. Activating histone proteins were globally downregulated in the cortex whereas the repression mark was increased at the end of life.

Manuscript III, utilized the innovative technique of ChIP-seq to identify H3K9Ac binding peaks in the mouse genome across the lifespan of developmentally Pb exposed mice. Unique peaks were present in the Pb exposed animals, with an emphasis on peaks in the promoter were reported. Of the genes affected, it was found many were associated with AD pathology by utilizing the Kegg's pathway thingy, specifically it was shown that genes have acetylation islands in or near the promoter that were differentially acetylated in Pb exposed mice later in life. Therefore, by these findings show that epigenetic regulation, specifically by H3K9Ac, could be a potential mechanism by which AD-related genes become upregulated late in life.

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I’m grateful to my family for providing valuable support and encouragement throughout this process. I would never have been able to get through these last four years without their love. Lastly, I would like to thank my friends for reminding me that it’s important to step back, take breaks and enjoy and love everything you do.

DEDICATION

To the boy with the greatest ambition I've ever known. You have, and will aways continue to inspire me.

Dave

PREFACE

This dissertation was prepared following the manuscript format. It has been divided into three manuscripts that relate to the effects of lead exposure on epigenetic marks and regulators. The first manuscript is a review article on the consequences of lead exposure in human populations, its relevance to neurodegenerative disease and the possibility of epigenetic involvement in this process, it has been accepted and published in the journal of Neurotoxicology. The second manuscript examines the alterations that occur to DNA methylation regulators and histone marks following developmental exposure to mice across the lifespan, this manuscript has been accepted and published in Alzheimer's and Dementia: Diagnosis and Disease Monitoring Journal. The final manuscript identifies the effects of lead on a specific histone mark, H3K9Ac at various timepoints in both control and lead exposed mice across the lifespan, this manuscript has been prepared following the Epigenomics journal guidelines.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

MANUSCRIPT I

Consequences of Lead Exposure, and it's Emerging Role as an Epigenetic Modifier in the Aging Brain

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Consequences of Lead Exposure, and it's Emerging Role as an Epigenetic Modifier in the Aging Brain

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ABSTRACT: Lead exposure has primarily been a concern during development in young children and little attention has been paid to exposure outcomes as these children age, or even to exposures in adulthood. Childhood exposures have long term consequences, and adults who have been exposed to lead as children show a host of cognitive deficits. Lead has also been shown to induce latent changes in the aging brain, and has been implicated in the pathogenesis of neurodegenerative diseases, particularly Alzheimer's Disease, and Parkinson's. Recent research has shown that lead has the ability to alter DNA methylation, histone modifications, and miRNA expression in experimental models, and in humans. These findings implicate epigenetics in lead induced toxicity, and long term changes in individuals. Epigenetic modification could potentially provide us a mechanism by which the environment, and toxic exposures contribute to the increased susceptibility of adult neurodegenerative disease.

1.0 Introduction

As we age our bodies grow increasingly susceptible to environmental injury and insults, the brain in particular is at great risk [1]. Interestingly, there aren't many toxicants that are discussed in the context of age related changes and abnormalities. A classic neurotoxin, that has been studied for it's role in childhood and developmental toxicity is the environmental agent lead (Pb). Historically, the toxic effects of lead have been researched and documented extensively as related to children and adolescents, however, the impact of past exposure to lead on the aging brain was not a major concern. There have been devastating cases of lead encephalopathy involving both adults and children, which is defined as a medical emergency, and observed when individuals have blood lead levels (BLLs) over 70 μg/dL [2]. Infants who have suffered acute exposure experience sever brain damage, and impaired neurological outcomes at doses even lower than what is considered lead encephalopathy (56 μg/dL) [3].

A dangerous property of lead is it's ability to interact and bind to calcium [4]. Over 95% of lead stores have been found to be deposited into bone, and it is considered a primary source of exposure. Measurements of both blood and bone lead levels provide researchers with evidence on how recent and past lead exposure may have occurred. Lead has been shown to be mobilized from the bone during periods of the human lifespan in which bone resorption/growth are occurring, for example during osteoporosis and pregnancy [5], giving lead the ability to induce toxic effects over prolonged periods of time, without recent

exposure. Lead has also been shown to compete with Zinc, in a number of physiological interactions. It has a similar affinity for motifs and receptors that are typically occupied by zinc, and ultimately is able to modify transcription. The effects of lead exposure on transcription, and it's dynamics with zinc have been extensively reviewed [6] .

In this review, we will discuss some of the classical outcomes as a result of lead exposure, but will focus on the role of lead in neurodegenerative and adult disease. We will introduce the role lead may have in regulating gene expression by way of epigenetics, and provide compelling evidence for lead as an epigenetic modifier.

1.1 Adult consequences of Childhood Exposure to Lead

Childhood exposure to environmental lead has been heavily implicated in cognitive dysfunction during early years. The toxicant has been identified as a clear disruptor of neurodevelopment in early life, shown to impair academic performance in school age children, and to negatively impact intelligence scores [7-13]. Furthermore, children who have unfortunately been exposed to lead during development have shown cognitive dysfunction that continues into adulthood. Early studies examined children who suffered from lead encephalopathy in the first four years of life, were found to have decreased scores on a battery of neuropsychological tests [14]. Similar findings were reported in a group of young adults (aged 19-29), who resided nearby a lead smelter facility during childhood [15]. More recently, a study conducted in Boston MA examined young adults (mean age 29) and their cognitive function,

by IQ tests. Individuals were known to have low-level (<10μg/dL) environmental lead exposure during childhood, and had measurements of blood lead taken at 6, 12, 18, 24 and 57 months, and again at 10 years of age [16]. The study reported lower IQ test scores in individuals with higher levels of exposure during childhood [16].

Longitudinal studies have been carried out to characterize the changes in brain development that are associated with this early exposure, namely in terms of brain volume reduction in specific regions. The Cinncinatti Lead Study (CSL) recruited a birth cohort from Cincinnati between 1979-1984, infants were excluded if they had low birth weight, or medical issues [17]. The CSL reported childhood BLLs were associated with regions of brain volume reduction in adult gray matter. Specifically this loss occurred in the prefrontal cortex, in regions associated with executive function control, behavioral modulation and fine motor control [18]. Furthermore, a subset of adults were recruited to high resolution volumetric magnetic imaging, and these changes were related to mean blood levels in the first six years of life. Significant inverse associates between age, gray matter volume and BLLs were observed, with the strongest reductions in adult gray matter associated with BLLs measurements at 5 and 6 years of age [19]. Further analysis of this cohort revealed significantly decreased levels of N-acetyl asparatate metabolite in gray matter as measured by proton magnetic resonance spectroscopy [20], these findings were replicated in a similar cohort [21].

These observations implicate lead in long lasting brain abnormalities that impact cognitive function negatively.

1.2 Exposures in adult populations

Evidence that exposure to lead is associated with cognitive decline is present from several longitudinal and cross-sectional epidemiological studies in the elderly. The onset of cognitive decline is an important intermediary for the development of neurodegenerative diseases, specifically Alzheimer's disease. The Baltimore Memory Study (BMS) was conducted to investigate determinants of cognitive decline while taking into account variables such as socioeconomic status, and environmental exposures [22, 23]. The cohort included individuals aged between 50-70 years, who lived in neighborhoods near Baltimore MD, and measured both blood and tibia lead levels [22, 23]. Results from the BMS indicated mean tibia levels were inversely correlated with cognitive function in all six domains tested, such as executive functioning, processing speed, and verbal memory and learning [22, 24]. Similar findings were reported by the Normative Aging Study (NAS) which began in 1963 and was conducted at the Veterans Affairs outpatient clinic in Boston, MA [25]. NAS enrolled 2,000 male veterans with the goal of investigating processes behind normal aging. They examined lead bone levels and results of the mini-mental state examination within this cohort, and reported higher bone levels are associated with worsened cognition [26, 27]. These findings were expanded in subsequent years, and the cohort was examined using a battery

of cognitive tests, such as the Wechler Adult Intelligence Scale- Revised results indicated a further decline in cognitive scores across all domains [28].

While most of the studies performed have focused on examining cognitive function in males, there are a small number that have contributed to our understanding of the effects of lead primarily on women. The Nurse's Health Study established in 1976 began to collect health information from registered nurses in the United States, the study has continued to monitor health outcome changes every two years until the present day, and has a participation of >90% of individuals since its establishment [29]. Weuve et al, reported on a subset of the Nurse's study, and examined blood, tibia, and patella levels of lead in relation to current cognitive function in community dwelling women. The study identified the three biomarkers of lead exposure were associated with worsened cognitive function in women, however only tibia levels were significantly higher [30]. These studies were replicated by others, where tibia levels were significantly associated with cognitive decline [31].

1.3 Occupational exposures

Due to our increased knowledge and awareness of the dangers of lead toxicity, exposures have been relatively controlled for most community dwelling individuals, while those exposed to lead in the workplace remain at risk. Both cross-sectional and longitudinal studies have been conducted in in workers exposed to lead, with studies occurring both in the United States and abroad. The Lead Occupational Study originally began in 1982, and examined

288 male workers with exposure to lead for a minimum of one year, while working at a lead battery plant in Pennsylvania. Cognitive functions were analyzed using the Pittsburg Occupational Exposures Tests (POET) [32]. POET results of this initial analysis found only significant associations between bone and BLLs and psychomotor speed [33, 34]. Members of this cohort were analyzed again to examine longitudinal changes in cognitive function. Khalil et al., reported that individuals who were reexamined had lower cognitive performance compared to control, as well as lower cognitive performance longitudinally. Unlike the initial study, the cognitive disruptions were observed between peak tibia lead levels, spatial ability, learning and memory and overall cognitive scores as determined by the POET battery test. Furthermore, when these results were examined by age it was determined that older individuals (>55 years) had more severe cognitive declines and dysfunctions than their younger counterparts [35].

These findings were also observed in the Organolead study, which began in 1994 to examine the effects of tetraethyllead manufacturing on cognitive functioning, based on earlier efforts from researchers at Johns Hopkins [36]. The cohorts last known lead exposure was 16 years prior, results indicated mean tibia lead levels were inversely correlated with neurobehavioral tests scores in the domains of manual dexterity, executive functioning, intelligence and memory [37]. Individuals were examined again two years later, with further associations of cognitive decline in relation to tibia lead levels in all areas [38]. Studies abroad have also focused on studying the

effects of lead exposure in the workplace. The Korea Lead Study began in 1997 and examined both current and former inorganic lead workers in the republic of Korea. The study employed 803 lead workers, and cognitive tests were employed similar to those in the organolead study, which were a modified version of the world health organization neurobehavioral core test battery [39]. Of these tests, higher lead blood levels, and not tibia lead levels were associated with worse performance on eight of the tests, which were associated with measuring executive functioning, and manual dexterity. Indicating BLLs as a better predictor of worse neurobehavioral scores [39]. The cohort was examined again following two years, measures at this time point showed BLLs consistent with earlier reports, with declines in both cognitive test scores related to manual dexterity and executive ability. Furthermore, individuals had worsened cognitive scores, consistent with cognitive decline associated with the cumulative lead exposure [40].

Functional associations between the cognitive deficits and anatomical brain changes have been undertaken by Stewart, Schwartz and colleagues on former organolead workers living in the eastern United States. Study participants were past organolead workers with 18 years mean time from their last exposure, and with a mean age of 56 years at time of enrollment. Bone lead concentrations were measured for individuals, and MRI data collected [41]. Cumulative lead dose was found to be associated with increased risk, and increased severity in white matter lesions**.** Investigators also observed total brain volume reduction, as well as reduction in specific brain regions such

as the cingulate gyrus, and insula. Effects observed by lead exposure were equivalent to reductions seen due to 5 years of aging [41]. Indicating that lead has a significant role in altering the architecture of the brain involved in cognition, and executive function and could potentially lead individuals to be more susceptible to the aging process.

2.0 Neurodegenerative Disease

It has been well established that as humans age, they become more vulnerable to a host of diseases, including cardiovascular disease, cancer and neurodegenerative disease [42, 43]. The nature for this is elusive to researchers, however there are some theories in the literature in regards to what may occur during the course of our lifetime, to make us more susceptible to these devastating diseases. Early life exposures and disease have been discussed since the 1980s, with the “Barker Hypothesis” that essentially states that adult diseases have a connection to the fetal environment, such as maternal stress and or diet. This early work primarily focused on adverse fetal events such as low birth weight, and their connections to the development of adult cardiovascular disease as the individual ages [44, 45]. Similarly, the developmental origins of health and disease (DOHaD) also describe the postnatal day period as a major window of vulnerability to adult latent disease, and just as important as the fetal environment [46].

It has been theorized that due to the accumulation of environmental stressors over the lifetime, that individuals become vulnerable to a host of neurological diseases. For example, the latent early life associated regulation

model (LEARn) describes a hypothesis in which environmental agents play a role in disease pathology by perturbing gene regulation in late life [47]. These environmental stressors are identified as “hits” that make individuals more susceptible to the development of neurological diseases as they age, typically by inducing epigenetic changes in the genome [47]. In a recent review, authors have described a number of exposure models that have contributed to the development of neurodegenerative disease, including *in utero* conditions, exposure to metals or pesticides, and dietary and lifestyle habits [48]. A number of studies have identified lead as a potential contributor, or risk factor in the development of neurodegenerative diseases. This section will focus on those insults that are associated with both development and occupational exposure to lead, pulling from both epidemiological and experimental data to demonstrate the potential role of lead in neurodegenerative diseases.

2.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that results in brain atrophy of cortical and hippocampal areas and is accompanied with the development of dementia. There are two pathological hallmarks of the disease, the amyloid beta (Aβ) plaque, and neurofibrillary tangle (NFTs), both of which are formed from the abnormal processing and accumulation of the amyloid precursor protein (APP) and microtubule associated protein tau (MAPT) respectively [49] .

While there is limited information regarding large-scale occupational and developmental exposure to lead in humans and the development of AD in

late life, there are some unique studies that have still managed to identify connections between exposure, and the disease pathology. The earliest example was obtained from the examination of a patient post mortem who survived a case of severe lead encephalopathy early in life, (2 years of age). The individual exhibited signs of mental deterioration, cortical, temporal and hippocampal atrophy. But most significantly, there was a presence of both neurofibrillary tangles, and senile plaques [50]. Studies of organolead workers 16 years following the post recent exposure identified strong declines in cognitive function, that were more associated with individuals with the apolipoprotein E4 (ApoE4) allele variant. APOE4 has been implicated as a potential risk factor in late-onset AD (LOAD) [51]. Most recently, lead exposure *in utero* has been implicated in AD [52]. Measurements in these individuals in young adulthood (28-30 years of age) showed elevated expression of 196 genes, specifically ADAM9, RTN9, LRPAP1 that are involved in the clearance and production of Aβ [52].

Previous studies in animals have shown developmental exposure to lead in early life results is an observed overexpression of AD-related proteins in late life. Initial observations were conducted in rodent models, with lead exposure during postnatal day (PND) 1-20. Analysis of gene and protein expression in late life identified an upregulation of APP, and the cleavage enzyme Beta-secretase 1 (BACE1), consequently higher levels of both cleavage products Aβ40 and Aβ42, which compromise Aβ plaques were also significantly higher [53, 54]. Further analysis of this cohort revealed that lead

also altered the expression of tauogenic proteins. Total tau, phosphorylated tau, and enzymes involved in tau phosphorylation were increased at the end of life in animals developmentally exposed; this was accompanied by cognitive deficits [55]. Results were further verified and reported in a primate model, with exposure to lead acetate (1.5 mg/kg/day) occurred from PND 1-400 [56-58]. Unlike rodents, primates are able to develop Aβ plaques and neurofibrillary tangles, and these animals showed increased presence of both pathological hallmarks in late life [56, 57]. Findings in primates implicate this developmental lead in the production of important pathological hallmarks of the disease, and strengthen the potential role for lead in the developmental basis of AD.

2.2 Parkinson's Disease

Parkinson's disease (PD) is a movement disorder, which is characterized by bradykinesia, rigidity, tremor and postural instability. Neuronal cell loss occurs in the substantia nigra, with primarily dopaminergic cells loss [59]. The first evidence that exposure to lead may contribute to the development of PD, was found in 1997 [60]. This pioneering study examined a group of patients with idiopathic PD, and their age matched controls at Henry Ford Medical Center in Detroit. Individuals, who had an occupational exposure of over 20 years of either lead-copper or lead-iron, were found to have greater risk for developing PD. Other studies measured levels of lead deposited in trabecular and cortical bone, and revealed that higher lifetime exposure to lead is associated with an increased risk of PD by more than two fold in

individuals [61]. These findings were replicated in a larger cohort of individuals, and tibia bone lead levels were significantly associated with an increased risk for developing PD [62].

2.3 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) or more commonly known as Lou Gherigs disease is a neurodegenerative disease that involves the degeneration of lower and upper motor neurons [63]. Initial observations in occupationally exposed individuals identified lead as a risk factor for the development of ALS [64]. Further observations in US veterans examined mobilization of lead from bone, and the potential association with an increased risk of ALS. The study concluded that elevated blood lead is associated with higher chances of the development of ALS regardless of both bone turnover, or δ-aminolevulinic acid dehydratase (ALAD) genotype. Ultimately, incidence of ALS was higher in those individuals with past lead exposure, and an increase in BLLs is associated with a 2-fold increase risk for ALS [65]. These findings were supported by a recent meta analysis consisting of nine case-controlled epidemiological studies of occupational lead exposure and ALS risk [66]. More recently, it has been investigated if individuals with single nucleotide polymorphisms such as the HFE-H63D, are at a greater risk for ALS when coupled with lead exposure. Investigators have associated patella lead levels with a higher incidence of ALS, and with the co-representation of this polymorphism, that is associated with iron overload and increased oxidative stress [67].

3.0 The Role of Epigenetics

The aberrant nature of sporadic neurodegenerative diseases, and the little to no genetic connection to their etiology points to an environmental component playing a role in their pathogenesis. The environment is believed to either be a driving factor, or to increase an individual's susceptibility to neurodegeneration with age [68]. The major mechanism by which environmental toxicants could be playing a role in neurodegeneration is via epigenetic control of gene expression. Epigenetics is the study of alterations, or changes in gene expression without altering the underlying DNA sequence. Environmental agents have been heavily studied in their ability to induce epigenetic changes relative to diseases such as cancer, but recently these observations have been made for neurological diseases.

DNA methylation is the most common type of epigenetic regulation studied. It is the process by which DNA methyltransferases (DNMTs) add a methyl group to the 5' position of cytosine in CpG rich regions, by way of the methyl donor S-adenosyl methionine (SAM) [69, 70]. Methylation patterns are maintained by DNA methyltransferases such as DNMT1, DNMT3a, and DNMT3b, and the functional consequence of the recruitment of methyl groups and repressive binding proteins at the promoter region of genes is association with repression of gene expression [71, 72]. The nucleosome is the major component of chromatin consisting of DNA base pairs wrapped around an octamer of histone proteins, composed of two copies of Histone 2A (H2A), H2B, H3 and H4 with modifications to the N-terminus tail typically occurring in

the form of methylation, acetylation and phosphorylation [73, 74]. Typically, acetylation at lysines on H3 and H4 are associated with regions of chromatin that are open to transcription, and typically indicate gene activation. Whereas histone methylation marks such as H3K27me3, and H3K9me3 are involved with gene repression. Both histone modifications and DNA methylation have been shown to work in tandem to regulate gene expression by altering the conformation and accessibility of chromatin regions [75, 76]. Epigenetic regulation is also maintained by noncoding RNA's, that bind to specific mRNA transcripts, and inhibit their transcription [77, 78]. The field of miRNA research is still new, and our understanding is primitive with comparison to DNA methylation, and histone modifications.

Epigenetic changes have been identified in a wide host of disease states. Primarily, this work was initially performed in oncology, with distinct DNA methylation patterns associated with cancer subtypes [79]. This work has also lead to the inclusion of DNA methylation patterns as potential biomarkers for a number of cancers [80, 81]. Epigenetics patterns, and epigenetic regulation of disease has also been observed in neurological disease states, including neurodegenerative diseases. In blood and brain measurements of PD patients, differential methylation patterns were observed in a number of genes relative to age-matched controls [82]. Observations in cellular models of ALS exhibited an upregulation of DNMTs and 5-methylcystosine (5MC). These findings were also observed in human ALS motor neurons, implicating DNMT proteins as potential drivers for neurodegeneration [83]. Hypomethylation was

reported at the APP promoter of an AD patient and consequently an upregulation of APP [84]. Further genome wide methylation studies in postmortem patients diagnosed with late-onset Alzheimer's disease revealed 948 CpG sites representing 918 genes that may be associated with this form of the disease. Further analysis will need to be performed to identify if the methylation status of candidate genes actually participate in the disease pathology [85].

Proteins involved in regulating histone and DNA dynamics have also been altered in Alzheimer's disease patients. A number of studies have identified increases in histone deacetylase proteins, such as HDAC6 and HDAC2 while DNMT1 levels were significantly reduced [86-88]. miRNA have also been found to be involved in neurodegenerative diseases. MiR107 is known to regulate BACE1 in human frontal cortex samples from AD patients. During the early pathogenesis of AD miR107 is upregulated and inversely related to BACE1, however as the disease progresses miR107 decreases, BACE1 levels are upregulated, and are involved in increased Abeta metabolism [89]. In PD patients, miR-34b and miR-34c were downregulated relative to age-matched control patients [90].

3.1 Lead as an Epigenetic Modifier

We have seen the emergence of a strong role for lead as a potential modifier of gene expression via epigenetic regulation, summarized in Figure 1. Some of the earliest information has come from the Zawia group, at the University of Rhode Island in their developmental studies of lead exposure in

an animal model of AD (discussed above). An early study examined both DNA methylation regulators and histone modifications in *macaca fasicularis* exposed to 1.5mg/kg/day of lead acetate from PND1-PND400. At 23 years of age exposed primates were found to have decreased levels of DNMT1, DNMT3a, and MeCP2 proteins involved in regulation DNA methylation, as well as significantly lower levels of acetylated histones [58]. In the same cohort of animals, DNMT1 activity was also lowered following lead exposure *[56].* *S*imilar findings were also observed in differentiated SHSY5Y cells. Human neuroblastoma cells were treated with increasing concentrations of lead acetate, and DNMT proteins and MeCP2 were significantly decreased *[91].*

Lifespan studies in mice have revealed genome wide dysregulation of DNA methylation in latent life following exposure. Methylation and gene expression profiles were overlaid and analyzed in animals at PND20 and PND700 following exposure, the results indicated a global repression profile of genes in late life, with a small subset of genes being overexpressed [92, 93]. Data from the Zawia lab have shown significant decreases of DNMT1, and MECP2 across the lifespan of wild type mice following developmental exposure to lead [94].We have also identified upregulation of H3K9Ac, and downregulation of H3K27me3, indicating that lead has the ability to reprogram the epigenome across the lifespan [94].Further studies in this same cohort identified a dysregulation in miRNA across the lifespan a well, with significant changes in miRNA involved in AD pathology [95].

Studies from other groups have also identified lead as a disruptor of epigenetic control of gene expression. In mice, perinatal exposure to lead resulted in a downregulation of DNMT1 and MECP2 at PND 55 in the hippocampus [96]. In rats chronically exposed to lead, the expression of 7 miRNA were altered. These miRNA have been previously shown to regulate genes involved in neurodegeneration, and processes involved in synaptogenesis and neuronal injury [97]. Acetylated histone 3 levels were also significantly increased with lead concentration (4mg/L, and 25mg/L), and associated with an increased hyperactivity in rats [98]. Lead exposure in human embryonic stem cells has been shown to alter DNA methylation status of genes involved in neural signaling [99]. Further analysis by this group has revealed that early exposure can disrupt 5-hydroxymethylation (5hmC) at CpG clusters in both human embryonic stem cells and umbilical cord blood. Studies of lead exposure in occupational cohorts have also identified DNA promoter methylation changes, individuals showed hypomethylation for LINE-1 [100], and hypermethylation of ALAD genes [101]. Umbilical cord blood was obtained from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) cohort and has potentially revealed both 5mC and 5hmC genomic loci that could be markers for prenatal lead exposure [102]. Lead induced changes in methylation have also been recently reported as transgenerational, with incredible data indicating that a mother's BLLs and methylation status, can directly impact that of her children [103]. This is the first work to indicate transmittable epigenetic regulation in response to an environmental toxicant.

4.0 Conclusion

The recognition of lead as a toxicant that endangers childhood welfare enabled many changes across the United States, and the world. Lead levels began to historically drop as the health dangers began to be recognized, leading to the current safety level of 5μg/dL [104] . These changes resulted in the eradication of lead based paint, as well as the removal of lead from gasoline [105, 106]. Despite these efforts lead exposure remains a prominent threat to children as well as adults across the world. In the United States, the current “safe” levels of exposure to lead are 5μg/dL for children. However, there have been studies to identify cognitive impairments at blood levels close to 5ug/dl, and even below, implying that there may not be any safe level of exposure [107-109].

Lead exposure still remains as a large risk for specific populations, specifically children. Unlike adults, children retain 32% of lead that is ingested, thereby making any exposure to lead that they encounter significantly dangerous [110]. Individuals living in urban areas, such as New Orleans are at great risk for exposure due to increased levels in the environment [111-113]. Despite regulations leading to the closing of lead smelters, areas and populations that have resided nearby continuously experience higher lead blood levels than are considered safe, and lead remains integrated in the environment. In 2012 a study examining BLLs in inner-city neighborhoods in Indianapolis IN reported 8% of children had BLLs higher than 10μg/dL [114]. With the passing of the new BLLs safety recommendation by the CDC, these

neighborhoods reported 27% of children with BLLs higher than the 5μg/dL [115]. In Flint MI a recent study has reported that 4.9% of children under the age of five had levels higher than the recommended 5μg/dL [116, 117]. Residential areas in developing countries are also still highly exposed to lead, with individuals utilizing equipment that is either made from lead, or living in environments where the drinking water is heavily contaminated [118-123].

We have presented information and arguments to explain the challenges that environmental lead exposure poses to the aging brain. We argue that lead toxicity occurs across the lifespan, and that developmental exposures have long term consequences. Typically adults have been lower of concern when it comes to classical lead toxicity due to the development of the blood brain barrier. Despite this, we have compiled evidence to suggest that even exposures that occur during adulthood are associated with devastating outcome. Therefore, lead remains a real and prominent threat as an agent that is involved in the pathology of adult disease. Not only does it contribute to cognitive deficits in both children and adults, it could potentially be involved in the etiology of several neurological diseases. The ability of lead to work by epigenetic processes and pathways presents as a major challenge for how scientists consider it's toxicological effects. As we continue to learn and uncover the role of epigenetic modifications in disease, we must be wary of agents that have been shown to exhibit epigenetic actions, and what the functional consequences could be.

Figure 1

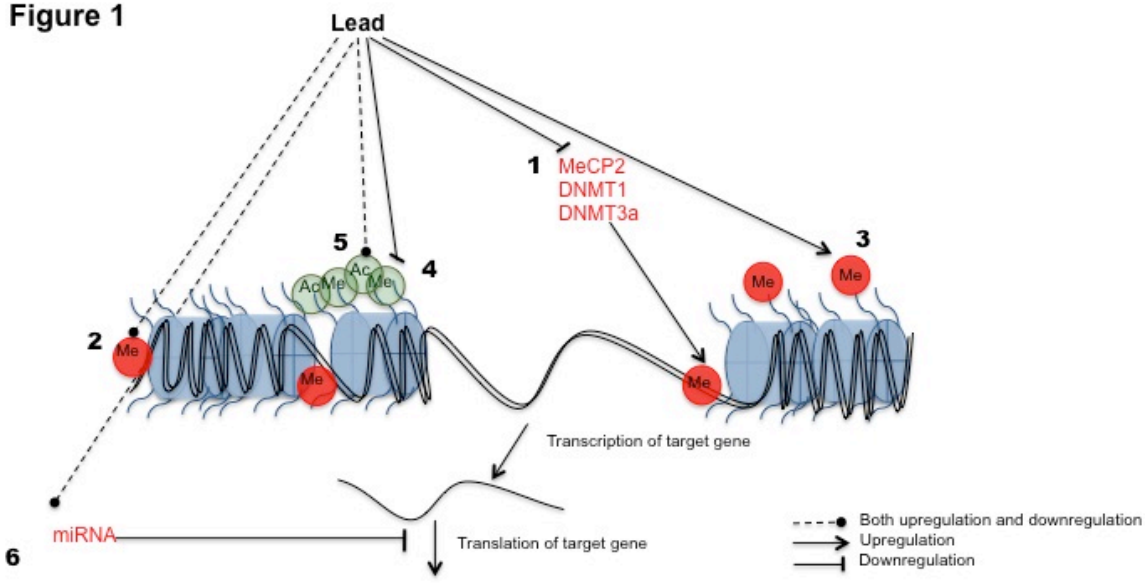
Lead
1
MeCP2
DNMT1
DNMT3a
2
3
4
5
6
Me
Ac
Transcription of target gene
miRNA
Translation of target gene
Both upregulation and downregulation
Upregulation
Downregulation

Figure 1: Epigenetic alterations following exposure to lead 1) Exposure has consistently shown to decrease MeCP2, and DNMT1 and DNMT3a levels in the literature. A decrease in these enzymes that govern DNA methylation is consistent with hypomethylation of genes. **2)** It has also been shown to both induce hypermethylation when analyzed by DNA methylation arrays, and also promoter specific hypo/hypermethylation by groups depending on the target gene being studied. Observations are consistent with upregulation of the repressive H3K27me3 **(3)**, and downregulation of active H3K4me2 **(4)**, whereas depending on the source and timing of exposure, lead induced upregulation/downregulation has been observed for H3K9Ac (5). Lead regulation of these mechanisms affects the transcript of the target gene. Whereas lead induced changes in miRNA expression **(6)** would either increase the availability of miRNA to bind to mRNA of the target gene resulting in a change in protein levels.

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MANUSCRIPT II

Developmental Lead (Pb) Exposure and Lifespan Alterations in Epigenetic Regulators and their Correspondence to Biomarkers of Alzheimer's Disease

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Developmental Lead (Pb) Exposure and Lifespan Alterations in Epigenetic Regulators and their Correspondence to Biomarkers of Alzheimer's Disease

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Abstract:

Background: Early life lead (Pb) exposure results in a latent increase in Alzheimer's disease (AD) related proteins, and cognitive deficits late in life in both rodents and primates. This study was conducted to investigate if these late life changes were accompanied by epigenetic alterations

Methods: Western blot analysis and RT-PCR were used to measure DNA methylation regulators (DNMT1, DNMT3a, MeCP2, MAT2A) and histone proteins (H3K9Ac, H3K4me2, H3K27me3).

Results: Cerebral levels of DNMT1 and MeCP2 were significantly reduced in mice exposed to Pb early in life, whereas the expression of DNMT3a was not altered. Levels of MAT2a were increased in the Pb-exposed mice across the lifespan. H3K9Ac and H3K4me2, involved in gene activation, were decreased, while the repressive mark H3K27me3 was elevated.

Conclusion: Epigenetic modifiers are affected by the developmental exposure to Pb, and may play a role in mediating the latent increases in AD related proteins in the brain.

Keywords: **Aging; Alzheimer's disease; Epigenetics; Lead (Pb); Lifespan**

I. Introduction

According to the 2015 Alzheimer's Report, there are currently 5.3 million Americans living with AD [1]. The vast majority of these individuals (>95%) are 65 years of age or older, with a disease subtype known as late-onset AD (LOAD). The remaining 5% of cases make up early-onset AD (EOAD), and of these (<1%) are linked to mutations in one of three genes, *Amyloid Precursor Protein* (*APP*), *Presenilin-1* (*PSEN1*), and *Presenilin-2* (*PSEN2*) [2]. LOAD is challenging to researchers, the presentation of the disease is sporadic as patients exhibit non-Mendelian characteristics. To date, there is no clear genetic etiology for LOAD, other than a risk factor of carrying specific susceptibility alleles [3, 4]. The single and only proven risk factor for the development of this sporadic form of AD is aging. These observations suggest the involvement of environmental and/or epigenetic factors across the lifespan in the initiation and development of the disease [5].

Early studies have provided evidence that many chronic adult diseases and disorders, such as cardiovascular disease, diabetes and obesity, are linked to environmental exposures that occurred during development [6, 7]. Importantly, there is also a growing body of literature to support the contention that exposures to environmental toxins in early life contribute to the development of at least some of the neurodegenerative disorders [8].

There are few epidemiological studies examining the link between environmental exposures and development of LOAD. These studies have assessed relationships between past exposures to pesticides, metals, dietary

changes and other environmental influences, however the results have been limited. We have taken a specific interest in the environmental agent and heavy metal lead (Pb). Since Pb has been implicated as a neurotoxin for generations, and is known to have devastating consequences for the nervous system, specifically in children [9]. The metal is non-degradable, ubiquitously found in nature, has high bioavailability, can cross the blood brain barrier and has a long half-life [10]. Epidemiological studies assessing both occupational exposure and exposures in community dwelling individuals have identified Pb as a clear disrupter of cognitive performance and function [11, 12]

Our lab was among the first to provide evidence that exposure to an environmental Pb in early life results in latent overexpression of AD-related proteins and histopathology in late life [13-16]. Reports by us have also demonstrated a latent increase in both amyloidogenic and tauogenic proteins, as well as behavioral deficits in our rodent models [13, 17]. These results have been replicated, and further investigated in a primate model, where we have also reported the presence of tangles and plaques in the cerebral cortex [14, 16]. More recently, we have shown that the changes in the expression of proteins such as APP, amyloid beta (Aβ) and microtubule-associated protein tau (MAPT) are accompanied by epigenetic changes [14, 18]. Epigenetics refers to the process that results in modifications in gene activity independent of the primary DNA sequence [19]. DNA methylation alterations and histone tail modifications are the most widely studied forms of epigenetic modifications.

This study documents the expression of four enzymes important in regulating DNA methylation and three histone modifications (two activating, one repressive) across the lifespan of animals that were exposed to Pb during a brief period of early postnatal life. Our results provide a valuable framework for understanding a number of epigenetic interactions that likely play a critical role in the expression of neurotoxic AD-related proteins in later life. This work is the first to identify epigenetic changes across the lifespan following a developmental exposure that has been previously shown to have significant increases in AD related biomarkers.

2. METHODS

2.1 Animal Exposure:

C57BL/6 mice were bred in house in the Animal Care Facility at the University of Rhode Island, according to previously published protocols [15, 17]. All experiments were performed in accordance with the standard guidelines and protocol approved by the University of Rhode Island Institutional Animal Care and Use Committee (IACUC) with supervision of the university's veterinarian. Males were divided into two groups, and used in this study. The control group received regular tap water, whereas the second group (PbE, developmental exposure) was exposed to 0.2% Pb-acetate from PND 1 to PND 20 through the drinking water of the dam. Animals receiving Pb-acetate had no observable adverse developmental deficits. Brains were dissected at the following ages: PND 20, 180, 270, 540 and 700 and stored at -80°C until use. These animals are from the subset of the same cohort that

has already been characterized for Alzheimer's pathology and behavioral deficits [15, 17].

2.2 Total RNA isolation, synthesis of cDNA, and real time PCR

Total RNA was isolated from the cerebral cortex following the TRIzol method (Invitrogen, Carlsbad, CA). Samples were checked for integrity and purity by NanoDrop (Thermo Fischer Scientific, Waltham MA). First strand complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Approximately 1 μg of RNA was diluted in 19 μl of water, with 4 μl 5x iScript reaction mix, and 1 μl of iScript reverse transcriptase. Samples were incubated at 42°C for 90 minutes, followed by 85°C for 5 minutes using the MJ Research MiniCycler PTC-150 (Bio-Rad). Real-time PCR was carried out in 12.5 μl reaction volumes containing 1 μl of cDNA template, 4.75 μl nuclease free water and 6.25 μl SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the ViiA 7 Real-Time PCR System (Applied Biosystems) utilizing the following conditions: 50 ^{o}C for 2 minutes followed by 95 ^{o}C for 10 minutes then 40 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute. Expression data was reported using the $2^{-\Delta\Delta CT}$ method and GAPDH mRNA as the endogenous control. The primer pairs used are presented in Table 1.

2.3 Protein Extraction

For DNMT1, DNMT3a, and MECP2 the nuclear fraction was collected according to the method described by Dignam et al, with minor modifications [20]. Samples were homogenized in 1 ml phosphate buffer saline (PBS) at pH

7.4 followed by centrifugation at 2500x g for 10 minutes. The pellets obtained were resuspended in 5 volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 0.2 mM PMSF) and centrifuged at 600x g for 2 minutes at 4°C. The pellets were resuspended in 3 volumes of buffer A and centrifuged at 600x g for 2 minutes at 4°C. Following centrifugation, pellets were then resuspended and homogenized in 5 vol of buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 420 mM NaCl, 20% glycerol, 0.2 mM PMSF, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin). Suspensions were centrifuged at 12,000x g for 10 minutes at 4°C, supernatants were collected, frozen and stored at -80°C until use. Cytoplasmic protein samples were isolated using the NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's instructions (Thermo Fischer Scientific).

2.4 Histone Extraction

Total histones were extracted using the EpiQuick Total Histone Extraction Kit (Epigentek, Farmingdale, NY) following the manufactures instructions, with minor modifications. Cortical tissue was weighed and cut into 1-2 mm pieces and homogenized at 200 mg/ml with prelysis buffer. The homogenate was centrifuged at 10,000 rpm for 1 minute at 4^{o}C. The supernatant was discarded, and the pellet was resuspended in 3x volume of lysis buffer and incubated on ice for 30 minutes. The sample was centrifuged at 12,000 xg for 5 minutes at 4^{o}C and the supernatant was transferred to a fresh vial. Balance buffer was

added to the supernatant (0.3 mL to 1 mL supernatant). The extract was placed at -80°C for long-term storage.

2.5 Western Blot Analysis

Protein concentration was determined by using the Micro BCA Protein Assay Kit (Thermo Fischer Scientific). Protein samples were then denatured and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 1-2 hours, and then transferred to polyvinyl diflouride (PVDF) membranes (GE-Healthcare, Piscataway, NJ), blocked for 1 hour with 5% bovine serum albumin (BSA) and then immunoblotted with the appropriate primary antibody. For MAT2A, 40 μg of cytoplasmic extracts were separated on 10 % SDS-PAGE gels and incubated overnight with primary polyclonal antibody at the dilution 1:1000 of ab77471 (Abcam, Cambridge, UK). Membranes were then stripped and reprobed with 1:2000 dilution of monoclonal A3854 (Sigma-Aldrich, St. Louis, MO) for β-actin. For DNMTs and MeCP2 proteins, 100 μg of nuclear extracts were separated on 5% SDS-PAGE gels and incubated overnight with primary monoclonal antibodies (Cell Signaling, Danvers, MA) 1:1000 dilution of D59A4 for DNMT1, D23G1 for DNMT3a and D4F3 for MeCP2. Membranes were then stripped and reprobed with 1:2000 dilution of G8795 (Sigma-Aldrich, St. Louis, MO) for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For histone proteins, the following primary polyclonal antibodies (Epigentek) were employed at a 1:5000 dilution, A-4022 for H3K4me2, A-4022 for H3K9Ac, A-4039 for H3K27me3, A-4035 for H3K9me3 and A-1112 for total H3. 10μg of histones

were separated onto 16% gels and incubated overnight with the respective primary antibody. Membranes were then washed four times with tris buffered saline with Tween 20 (TBST), and incubated with appropriate infrared-labeled secondary antibody (Li-Cor, Lincoln, NE) at 1:10000 for 1 hour at room temperature in the dark. Infrared band signals were detected and quantified using an Odyssey Infrared Imaging System (Li-Cor). MAT2A protein was normalized against β-actin, whereas nuclear proteins were normalized against levels of the housekeeping protein GAPDH. Histone proteins were normalized against bands for total H3.

2.6 Statistical Analysis and Data Representation:

Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). Real-time PCR analysis was completed using the applied biosystems ViiA7 software. Each data set has either an n=3, or n=4 (designated in the figure legend), the animals were measured independently without pooling of brain tissue or samples. Individual data points are presented on each graph, as well as the mean for each group. The vertical bars represent 95% confidence intervals and are shown for each data set. The significance of difference between different treatment groups was determined by one-way analysis of variance (ANOVA), with the Holmes-Sidak post hoc test using IBM SPSS statistics 21 software. The significance of interaction between treatment groups and time was determined by two-way analysis of variance. The level of significance was set at $\alpha=0.05$. The change in protein levels with respect to each time point were analyzed by ANOVA with

the Dunnetts post hoc test.

3. RESULTS

We have previously measured Pb concentrations in animals exposed under the same protocol as the one in this manuscript and found the concentration of Pb in the cerebellum of PND 20 rodents (0.25 +/- 0.07 μg/g) to be approximately three times the level seen in control animals [21]. Blood levels have been shown to be 46.43 μg/dl during Pb exposure but are reduced to basal levels in adults [13]. The current recommended levels by the CDC is that of 5 μg/dL, even though the blood levels in our exposure scenario are higher they are still consistent with the levels seen in both children and adults exposed to environmental Pb [22, 23].

3.1 Effect of Pb exposure on the expression of DNMT1 and 3a protein

There was no significant interaction effect between Pb treatment and time. The exposed mice had a statistically significant decrease in normalized DNMT1 levels at all time points across lifespan relative to the control animals. In both the control group and Pb-exposed animals DNMT1 protein expression was decreased at all time points relative to PND20. (Figure 1.A). Unlike DNMT1 protein levels, DNMT3a showed no significant difference between control and Pb-exposed animals across the lifespan of the mice (Figure 1.B).

3.2 Levels of MeCP2 protein across the lifespan following exposure

MeCP2 protein levels normalized relative to GAPDH are increased at day 270 in both the control and Pb-exposed animals; however these results are

not significant. Statistically significant decreases between the Pb exposed group and the control group are observed at day 180, and 540 as seen in Figure 2.A. The remaining time points exhibited no significant difference between Pb exposed and controls. The control group exhibited no differences in proteins levels from PND 20 at any other time point. Whereas in the Pb-exposed animals, there was significant differences in protein expression observed at all timepoints except for PND 270. There was also no significant interaction effect between Pb treatment and time.

3.3 Protein levels of MAT2A protein across the lifespan and following Pb exposure

The cytoplasmic protein expression profile of MAT2A across the lifespan of control groups did not display any significant change (Figure 2). However, mice exposed to Pb as infants demonstrated a statistically significant increase in levels of MAT2A at day 270, 540, and 700 relative to control. Similarly there is no interaction effect between time and treatment.

3.4 Alterations in gene expression of DNA mediators following Pb exposure

Changes in gene expression of *Dnmt1, Dnmt3a and Mecp2* were also examined by Real-Time PCR analysis. We observed no significant difference between mRNA levels of *Dnmt1,* across the lifespan between the control and Pb exposed animals (Figure 3.A). *Dnmt3a* mRNA levels for the Pb exposed animals were elevated compared to controls across the lifespan, and significantly increased at PND 20 (Figure 3.B). In figure 4.C mRNA for *MeCP2* is significantly lower at PND 20 and PND 270 for the exposed group, with no

change at any other time point. There was no significant interaction effect for any of these mRNA targets.

3.5 Effect of Pb on activating and repressive histone marks across the lifespan

Histone extracts were used to profile histone modification marks across the lifespan via western blot. The activating marks are shown in Figure 4. H3K9Ac protein levels were significantly lower in the Pb exposed mice for all time points except PND 20, as seen in Figure 4.A. In the control animals, there was significant differences compared to PND 20 at both PND 270 and 540, and in the Pb-exposed animals significance was observed at all timepoints relative to day 20. H3K4me2 levels have a similar trend, the Pb exposed mice have lower protein levels compared to controls across the lifespan, with the only significant decrease occurring at PND 20 (Figure 5.B). Similarly, there were no significant changes at any of the time points relative to day 20 in either the control group, or Pb-exposed group. Representative blots for H3K9Ac and H3K4me2 are seen in Figure 4.C, with total Histone H3 as a loading control. Protein levels for H3K27me3 are shown in Figure 5. H3K27me3 is a histone mark indicative of gene repression. Pb exposed animals showed significantly higher levels of H3K27me3 across the lifespan except for day 180. In the control animals, all time points exhibited significant differences relative to PND20, whereas in the Pb-exposed animals significance was only observed at PND 270 and PND 540. A representative blot for H3K27me3 is shown in Figure 5.B.

DISCUSSION

Epigenetic regulation is a complicated phenomenon with converging pathways involved in the regulation of gene expression. DNA methylation is maintained by DNA methyltransferases such as DNMT1, DNMT3a, and DNMT3b, and involves the recruitment of MeCP2 as well as other proteins[24]. Typically recruitment of methyl groups and methyl binding proteins is indicative of a decrease in gene expression, and is associated with repressive complexes [25]. Histone tail acetylation is associated with regions of chromatin that are open to transcription, and typically indicate gene activation. Whereas specific histone methylation marks such as H3K27me3, and H3K9me3 are associated with regions of condensed chromatin, and gene repression.

This *in-vivo* study uncovered a significant decrease in DNMT1 levels relative to their age-matched controls across the lifespan of mice developmentally exposed to Pb , with a significant decrease in MeCP2 levels at some time points as well. These findings are consistent with our previously published results indicating that differentiated SHSY5Y cells treated with increasing concentrations of Pb have a significant decrease in DNA modification enzymes, and MeCP2 [18]. DNMT1 is an important regulator of tissue specific DNA methylation patterns; exposure to metals such as Cd have been shown to alter the activity and protein levels of this enzyme [26]. We have also previously examined the ability of Pb to alter the activity of DNMT1 in tissue, and have found that exposure to Pb does decrease DNMT1 activity [14]. Studies from other groups have also examined changes in protein levels of DNMT1, and MECP2 as a result of postnatal and perinatal Pb

exposure [27]. They reported a downregulation in these DNA methylation modifiers in the hippocampus at PND 55 following Pb exposure. Our results are consistent with their findings, indicating that Pb exposure down-regulates these proteins in the cerebral cortex, and that this repression is never recovered. The data points collected in our study indicate that even at PND 700 there is downregulation of DNMT1, and MeCP2 is maintained.

The results obtained from studying the gene expression of these DNA methylation regulators leads us to believe that Pb is most likely decreasing their protein expression directly, and perhaps not through transcriptional regulation. There was no significant change in mRNA levels for DNMT1, but there was a decrease in protein relative to controls across the lifespan. A possible mechanism for this may be due to increases in protein degradation of DNMT1 following exposure. Similarly, MeCP2 mRNA was only significantly lower at the PND 20 and PND 270 time point. The most interesting observation obtained from the gene expression data is for DNMT3a. DNMT3a gene expression for the Pb treatment was higher across the lifespan, and significantly increased at PND 20, whereas the protein levels were not significantly different in the lead exposed animals relative to control. These data are explained by a recent finding from our lab showing that following exposure to Pb, as well as an increase in miR-29b (targeting DNMT3a) at PND20, suggesting that the miRNA leads to a degradation of DNMT3a mRNA and subsequently why we don't observe a change in DNMT3a protein levels [28]. The same paper reported an is an increase of miR-106 (Targeting APP)

at PND 700 [28]. There is also evidence that miRNA expression is effected by chronic Pb exposure in an adult rat hippocampus [29]. Our observations with DNMT3a are also consistent with previously published work by our lab indicating that DNMT3a gene expression is 2 fold decreased at PND 700 for the Pb treated group as compared to PND 20 in primates [30].

MAT2A catalyzes the formation of S-adenosylmethione (SAM) from methione and ATP and is an important regulator of the methylation cycle. It has been well documented that SAM levels have been found to be significantly decreased in the cerebrospinal fluid of patients with AD [31]. In our study we have shown that Pb exposed mice have elevated levels of MAT2A across the lifespan compared to their age matched controls. A recent study in hepatoma cells has shown that upon hypoxia exposure, DNA demethylation pathways are activated whereas MAT2A expression is up-regulated. [32]. In order to better understand the role of MAT2A and the changes of gene expression we will have to further investigate it's role in regulating the DNA methylation pathway in our model.

Histone modifications refer to additions of chemical groups to N-terminus tail of histone proteins, most commonly the histone core proteins, H3 and H4. Histone acetylation levels are governed by histone acetyltransferases (HATs) and deacetylases (HDACs), whereas histone methylation by histone methyltransferases (HMTs). It is known that histone modifications and DNA methylation interact to alter the conformation, and thus the accessibility, of the promoter and coding regions of the genome [33]. In this study, we have shown

that early life Pb exposure was found to decrease H3K9Ac, and H3K4me2 proteins levels, both of which are marks for gene activation. We have also shown that H3K27me3 levels are increased, which also is indicative of gene repression. These are consistent with our previous findings in our primate tissue, where we observed that histone repressive marks were upregulated, and the majority of genes are downregulated following Pb exposure[34].

Although much information can be gained from understanding the changes in the regulation of DNA methylation enzymes and proteins, we are limited in that we have not identified specific genes that are directly affected in the alteration of binding of these modifiers. We are also trying to investigate the mechanisms by which Pb exposure may contribute to the dysregulation of these proteins. It appears from these studies and previous studies, that following Pb exposure there are groups of genes that are upregulated, and others that are repressed. This upregulation of specific genes may be facilitated by DNA hypomethylation, whereas the gene repression may be histone-mediated. The changes that have been previously reported in this animal model report increases in AD related proteins latent in life, whereas the dysregulation observed in the epigenetic regulators occurs throughout the lifespan [15, 35]. Future studies will examine the alterations in DNA methylation, as well as histone modifications that occur at specific genes and the consequences that has for the expression of those genes. The implications of this work and the findings obtained from studying histone modifications and DNA methylation are vast. Targeting epigenetic marks is becoming recognized

as a novel therapeutic approach for neurodegenerative disease, including AD [36]. Treatments that have been explored include histone deacetylase (HDAC) inhibitors, such as valproic acid, and S-adenosylmethionine supplementation as a method to target DNA methylation changes [36].

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exposure to environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28:3-9.

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Tables and Figures:

GENE	FORWARD PRIMER	REVERSE PRIMER
DNMT1	5'-GAGTCTTCGACGTCACACCA-3'	5'-AGCTACCTGCTCTGGCTCTG-3'
DNMT3a	5'-CTTGGAGAAGCGGAGTGAAC-3	5'-GGATTCGATGTTGGTCTGT-3'
MeCP2	5'-CAGCAGCATCTGCAAAGAAG-3'	5'-TCCACAGGCTCCTCTCTGTT-3'
MAT2A	5'-ACCCTATGCATGGTTTCAGC-3'	5'-ACCCTGGGAGGAGCTATTG-3'
GAPDH	5'-AGGTCGGTGTGAACGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'
B-ACTIN	5'-TGTTACCAACTGGGACGACA-3'	5'-TCTCAGCTGTGGTGGTGAAG-3'

Table 1: Primer sequence pairs used in the study for the analysis of gene expression.

Figure 1

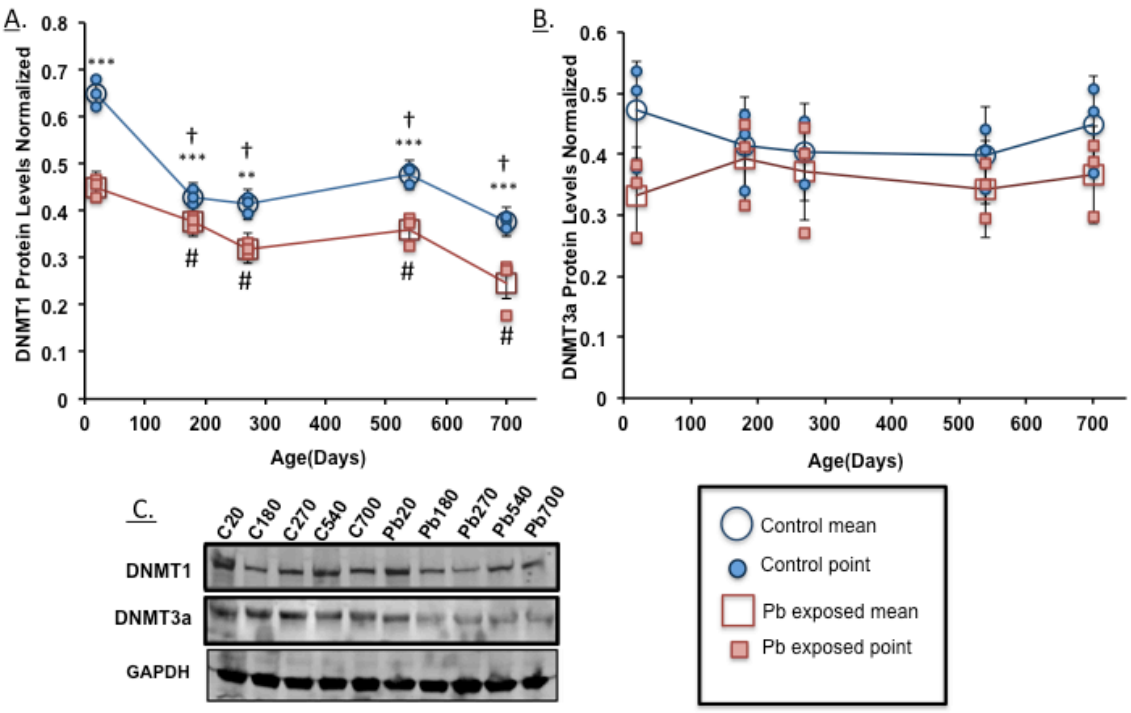
A.
DNMT1 Protein Levels Normalized
Age(Days)
B.
DNMT3a Protein Levels Normalized
Age(Days)
C.
C20
C180
C270
C540
C700
Pb20
Pb180
Pb270
Pb540
Pb700
DNMT1
DNMT3a
GAPDH
Control mean
Control point
Pb exposed mean
Pb exposed point

Figure 1: DNA methyltransferase (DNMT) protein levels across the lifespan following developmental exposure to lead (Pb). (A) Quantification of DNMT1 protein expression levels across the lifespan for control animals and those developmentally exposed to Pb. (B) Quantification of DNMT3a protein levels across the lifespan following developmental Pb exposure. (C) Representative DNMT1 and DNMT3a proteins levels. Nuclear extracts were used and proteins were measured by western blot analysis as described in the methods section. Individual data points are represented on the graph, as well as the means for each time point. N=3 and significance is represented as *P<0.05, ** P<0.01, *** P<0.001. † is used to denote significance from the PND20 group for the control animals, while # is used to denote significance from the PND20 time point for the lead exposure group.

Figure 2

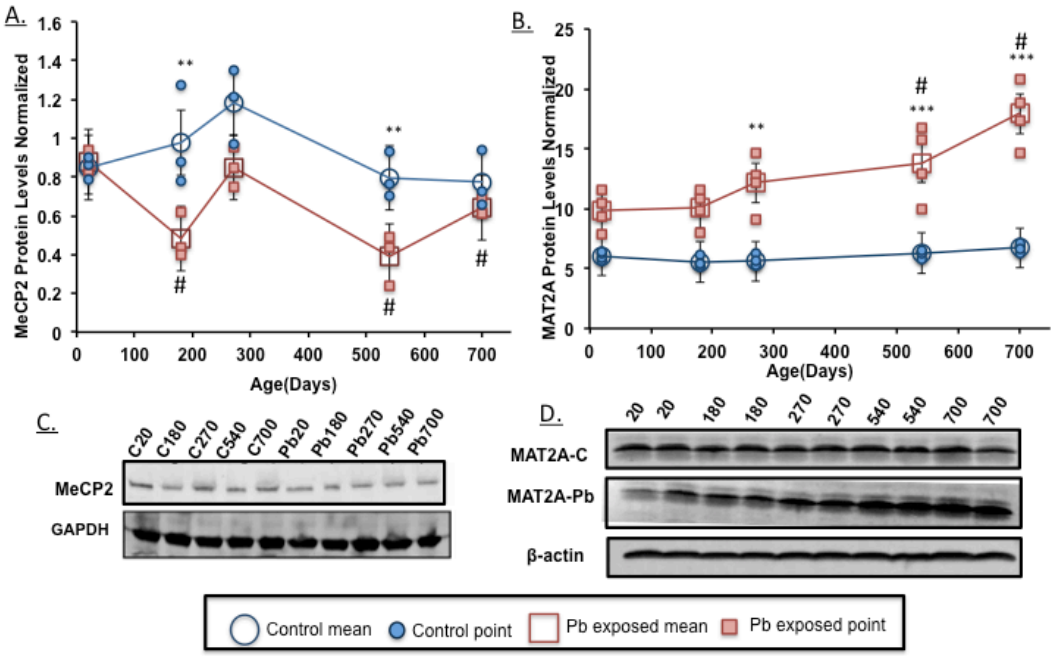
A.
MeCP2 Protein Levels Normalized
Age(Days)
B.
MAT2A Protein Levels Normalized
Age(Days)
C.
C20
C180
C270
C540
C700
Pb20
Pb180
Pb270
Pb540
Pb700
MeCP2
GAPDH
D.
20
20
180
180
270
270
540
540
700
700
MAT2A-C
MAT2A-Pb
β-actin
Control mean
Control point
Pb exposed mean
Pb exposed point

Figure 2: Methyl CpG binding protein 2 (MeCP2) and Methionine adenosyltransferase 2a (MAT2a) levels across the lifespan following developmental exposure to lead (Pb). (A) Quantification of MeCP2 protein expression levels across the lifespan for control animals and those developmentally exposed to Pb. (B) Quantification of MAT2A protein expression levels across the lifespan for control animals and those developmentally exposed to Pb. (C and D) Representative MeCP2 and MAT2A protein levels. Nuclear and cytoplasmic extracts were used and proteins were measured by western blot analysis as described in the methods section. Individual data points are represented on the graph, as well as the means for each time point. N=3 for MeCP2 and N=4 for MAT2A and significance is represented as *P<0.05, ** P<0.01, *** P<0.001. † is used to denote significance from the PND20 group for the control animals, while # is used to denote significance from the PND20 time point for the lead exposure group

Figure 3

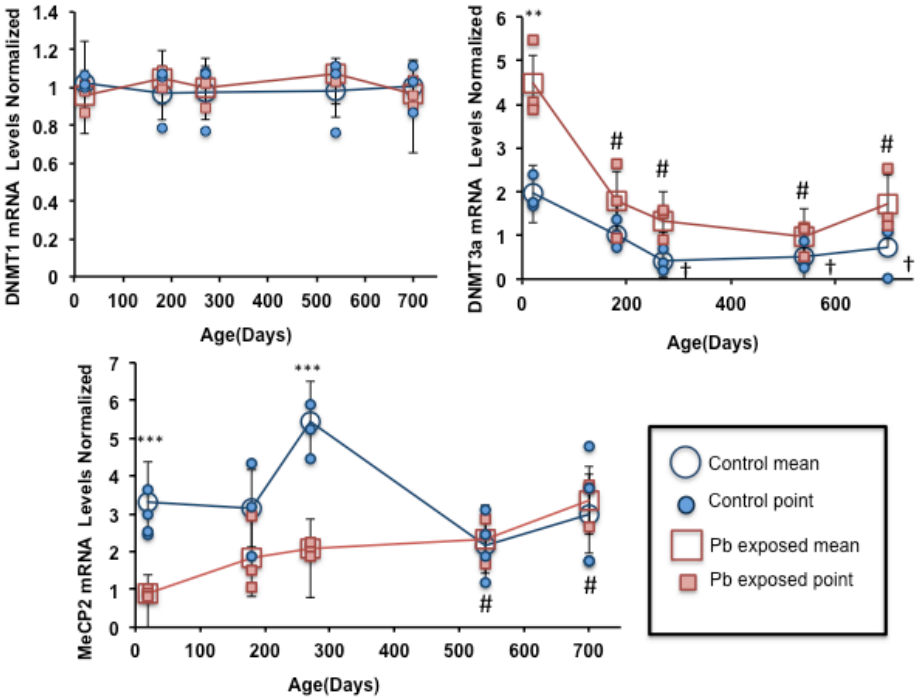
DNMT1 mRNA Levels Normalized
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 100 200 300 400 500 600 700
Age(Days)
DNMT3a mRNA Levels Normalized
6
5
4
3
2
1
0
**
#
#
#
#
†
†
†
0 200 400 600
Age(Days)
MeCP2 mRNA Levels Normalized
7
6
5
4
3
2
1
0
***
***
#
#
0 100 200 300 400 500 600 700
Age(Days)
Control mean
Control point
Pb exposed mean
Pb exposed point

Figure 3: mRNA levels of DNA methylation regulators across the lifespan following developmental exposure to lead (Pb). (A) Quantification of DNMT1 mRNA across the lifespan for control animals and those developmentally exposed to Pb. (B) Quantification of DNMT3a mRNA across the lifespan for control animals and those following infantile exposure. (C) Quantification of MeCP2 mRNA across the lifespan for control animals and those developmentally exposed to Pb. Individual data points are represented on the graph, as well as the means for each time point. N=3 or N=4 and significance is represented as *P<0.05, ** P<0.01, *** P<0.001. † is used to denote significance from the PND20 group for the control animals, while # is used to denote significance from the PND20 time point for the lead lead exposure group.

Figure 4

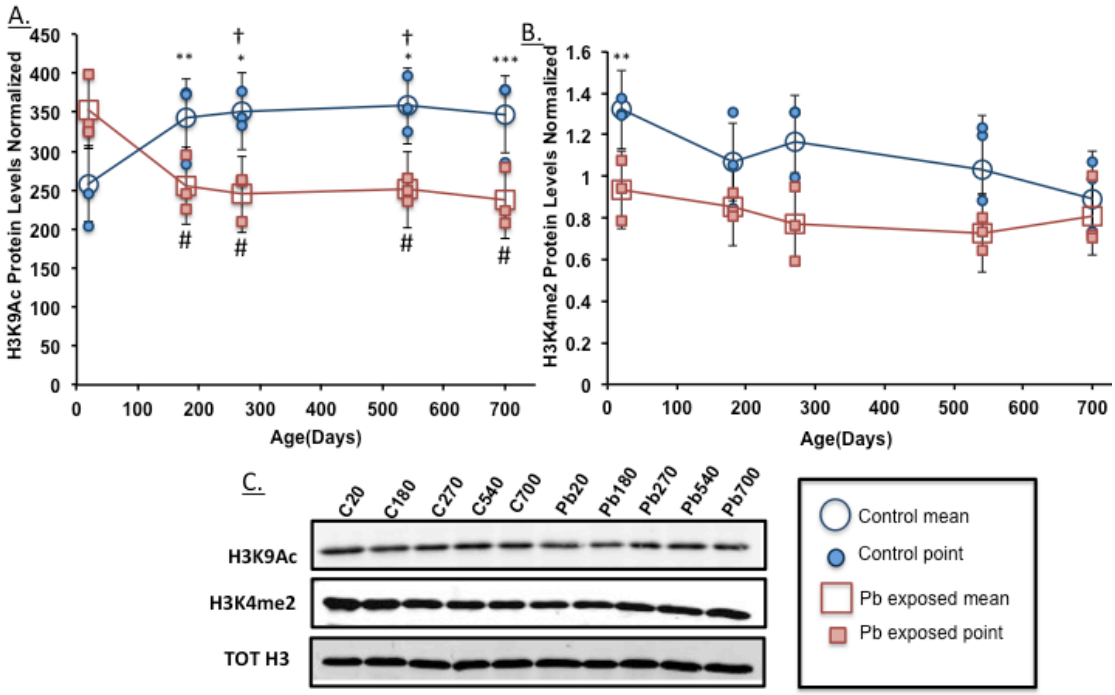
A.
H3K9Ac Protein Levels Normalized
Age(Days)
B.
H3K4me2 Protein Levels Normalized
Age(Days)
C.
C20
C180
C270
C540
C700
Pb20
Pb180
Pb270
Pb540
Pb700
H3K9Ac
H3K4me2
TOT H3
Control mean
Control point
Pb exposed mean
Pb exposed point

Figure 4: Activating histone protein levels across the lifespan following developmental exposure to lead (Pb). (A) Quantification of H3K9Ac protein expression levels across the lifespan for control animals and those developmentally exposed to Pb. (B) Quantification of H3K4me2 protein levels across the lifespan following developmental Pb exposure. (C) Representative histone proteins levels. Histone extracts were used and proteins were measured by western blot analysis as described in the methods section. Individual data points are represented on the graph, as well as the means for each time point. Individual data points are represented on the graph, as well as the means for each time point. N=3 and significance is represented as *P<0.05, ** P<0.01, *** P<0.001. † is used to denote significance from the PND20 group for the control animals, while # is used to denote significance from the PND20 time point for the lead exposure group.

Figure 5

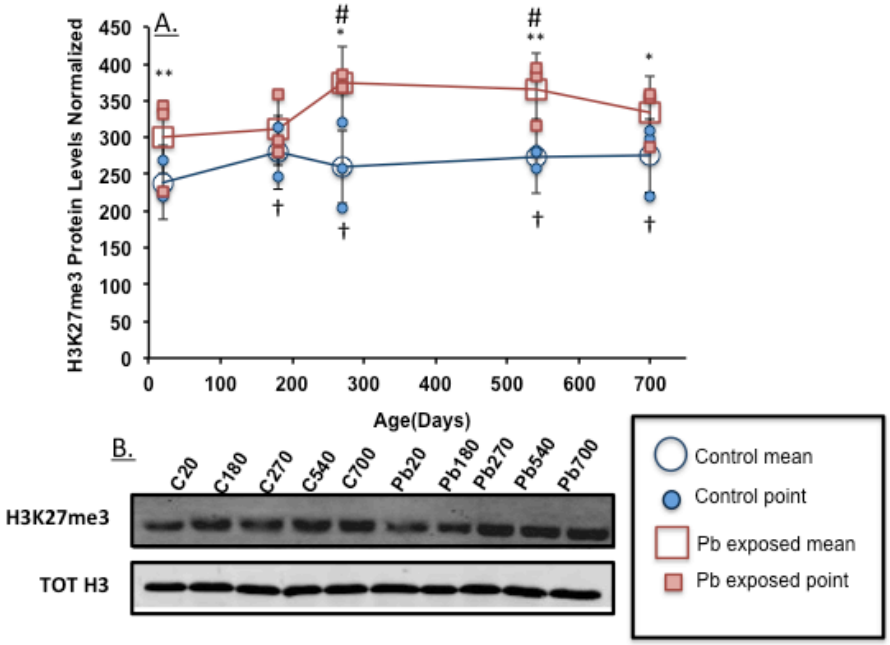
A.
H3K27me3 Protein Levels Normalized
450
400
350
300
250
200
150
100
50
0
0
100
200
300
400
500
600
700
Age(Days)
B.
C20
C180
C270
C540
C700
Pb20
Pb180
Pb270
Pb540
Pb700
H3K27me3
TOT H3
Control mean
Control point
Pb exposed mean
Pb exposed point

Figure 5: Repressive histone mark levels across the lifespan following developmental exposure to lead (Pb). (A) Quantification of H3K27me3 protein expression levels across the lifespan for control animals and those developmentally exposed to Pb. (B) Representative H3K27me3 protein levels. Histone extracts were used and proteins were measured by western blot analysis as described in the methods section. Individual data points are represented on the graph, as well as the means for each time point. N=3 and significance is represented as *P<0.05, ** P<0.01, *** P<0.001. † is used to denote significance from the PND20 group for the control animals, while # is used to denote significance from the PND20 time point for the lead exposure group.

MANUSCRIPT III

Lifespan Histone Acetylation Profiles in Mice Developmentally Exposed to Pb

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(Prepared for Submission to Epigenomics)

Lifespan Histone Acetylation Profiles in Mice Developmentally Exposed to Lead (Pb)

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Abstract

Introduction: Early life exposure to lead (Pb) has been shown to increase biomarkers involved in Alzheimer's disease (AD) pathology in wild-type mice in late life. In this same cohort, we observed significant changes in both activating and repressing histone proteins.

Methods: Chromatin Immunopercipitation Sequencing (ChIP-seq) technology was used to map histone acetylation at lysine 9 (H3K9Ac) binding to the mouse genome. Using bioinformatics methods we were able to align sequence reads to the genome, and then filter and examine the unique peaks from the Pb exposed group at each timepoint.

Results: Developmental exposure to lead increases H3K9Ac at a number of gene promoters in animals previously exposed to Pb across the lifespan. A number of these genes have been implicated in AD pathology.

Discussion: The increase of H3K9Ac peaks at the promoter of genes at day 700 indicates that there is a high chance that they are upregulated. Specifically the presence of the activation mark at AD promoter supports our hypothesis for the role of histone proteins in our model.

Keywords: Alzheimer's Disease; Epigenetics; Histone Acetylation; Lead (Pb)

1. Introduction

Environmental exposures have long been postulated to play a pivotal role in both promoting, as well as preventing disease. Numerous studies identify positive habits such as a healthy diet rich in polyphenols, regular exercise, caloric restriction and others as safeguarding against most cancers, metabolic syndrome, and neurological disease [1, 2]. Conversely, exposures from toxins and toxicants have been adversely associated with disease states. Among these are an increased risk of type two diabetes as a result of exposure from food contaminants such as persistent organic pollutants (POPs)[3]. Recent findings have also shown significantly increased levels of the organochloride β-hexachlorocyclohexane (β-HCH) in patients with Parkinson's disease relative to their age-matched controls [4]. Similarly serum levels of DDE the metabolite of DDT were 3.8 fold higher in patients with Alzheimer's disease (AD) [5].

The critical window for exposure in adult diseases occurs during early development, including both perinatal and postnatal exposures. Evidence for an increased risk of disease states has been referred to as the developmental basis of disease, and has been tied to many adverse states including metabolic syndrome and neurological diseases [6-8]. Specifically in AD, where the vast majorities of cases are late-onset with no clear etiology, the environment is thought to play a major role [9]. One proposed mechanism by which the environment may alter the expression of genes and predispose to AD is via epigenetic pathways [9, 10].

Epigenetic mechanisms refer to changes in our genome that affect gene expression without altering the underlying DNA sequence. Of these, the most commonly studied are DNA methylation and histone modifications. DNA methylation involves the addition of methyl groups to the cytosine residues of DNA. Recruitment of methyl groups to CpG islands of genes is usually involved in silencing of that particular region of the genome [11-13]. In the cell, DNA is organized around histone proteins, and histone tails can be modified by a number of posttranslational modifications that will either repress or enhance gene expression. Both DNA methylation and histone modifications work by recruiting other chromatin binding proteins to alter the structure of chromatin and recruit either activating or repressive elements [14, 15]. Specifically histone acetylation is involved in active gene expression, and is associated with loose binding of the histone proteins to the DNA, which in turn allows inclusion of transcriptional machinery [14, 15].

Our lab has extensively shown that early life exposure to lead (Pb) can have significant impacts on behavior in mice, and that this exposure is also associated with an increase in proteins involved in Alzheimer's pathology. Specifically, we have reported statistically significant protein and mRNA levels of the amyloid precursor protein (APP) and microtuble associated protein tau (MAPT) in those animals exposed to Pb [16, 17] . These mice were also shown to have behavioral deficits at 18 and 24 months of age as measured by both the water maze and Y-maze [18]. Recently, this same cohort was found to have alterations of proteins involved in modifying epigenetic marks such as DNA

methyltransferases, as well as significant changes in epigenetic marks themselves; including significant decreases in total protein levels of H3K9Ac, an important histone mark involved in gene activation [19] .

In this study, we have used a novel approach to examine H3K9Ac binding across the genome. There are a limited number of papers that utilize Chromatin Immunoprecipitation Sequencing (ChIP-seq) in brain tissue, as well as across the lifespan. There are only a handful of studies that implement similar research designs. A recent study reported genome wide alterations in histone acetylation at sites H3K9 and H3K14 by using microarray analysis in a mouse model of Huntington's disease [20]. H4K12 was also examined using ChiP-seq technology following learning and memory experiments in mice [21]. To the best of our knowledge, this is the only study in which the active histone mark (H3K9Ac) was analyzed across the lifespan as well as following an early life developmental exposure.

In this manuscript we performed ChIP-seq analysis across the lifespan in animals previously exposed to Pb compared to their age matched controls. Tissue samples from three time points, PND 20, 270 and 700, were profiled for H3K9Ac binding.

2. Methods

2.1 Animal Exposure:

C57BL/6 mice were bred in house in the Animal Care Facility at the University of Rhode Island according to previously published protocols [19, 22, 23]. Postnatal day 1 (PND 1) was designated as 24 h after birth. Male pups from

different dams were pooled and placed randomly to different dams (10 mice/dam). The mice were divided into two groups, the control group received regular tap water, and the second group (PbE, developmental exposure) was exposed to 0.2% Pb acetate from PND 1 to PND 20 through the drinking water of the dam. Cerebellar tissue from mice aged PND 20, 270, and 700 were used in this study. The animals used in this study have been previously found to have Alzheimer's pathology as well as behavioral deficits late in life [16, 22, 23]. We have also shown alterations in epigenetic regulators as well as epigenetic marks including histone proteins following exposure to Pb [19]. Specifically we observed significant decreases in levels of H3K9Ac across the lifespan following exposure to Pb [19].

2.2 Tissue Fractionation and Shearing Optimization

Chromatin Isolation was performed using the reagents from the ChIP IT Express Kit (Cat # 53008, 53032 Active Motif, Carlsbad CA) with modifications for working with tissue samples. All solutions were prepared according to the manufactures instructions. 100 mg of cerebellar tissue was pooled from mice (n=5) and was homogenized in with 4 mL of phosphate buffer saline (PBS) (1 mL per 25 mg of tissue), using dounce homogenizer. The cerebellum was used due to its dense and abundant neuronal populations that would yield sufficient material for these experiments. Samples were then incubated with 180 µl of 37% formaldehyde and placed on a shaker at room temperature for 15 min, and centrifuged at 3500 rpm for 8 min at 4°C. The supernatant was discarded, and the pellet was resuspended with 10 mL of glycine stop fix solution and incubated

at room temperature for 5 minutes on a shaker. The sample was then centrifuged at 2500 rpm for 10 min at 4°C and pellet was washed with 1 mL ice cold PBS. Samples were later centrifuged at 2500 rpm for 10 min at 4°C. The pellet was resuspended with 1 mL ice-cold lysis buffer and incubated on ice for 40 min. Samples were then centrifuged at 5000 rpm for 10 min at 4°C to pellet the nuclei. The supernatant was discarded and 350 µl of ice-cold shearing buffer was added to resuspend the nuclei.

Samples were sonicated using a Branson sonifier 150 on level 2. Each sample was sonicated 4 times at 20 seconds with 40 second intervals while on ice. The samples were centrifuged at 1500 rpm for 10 min at 4°C and supernatant collected. A 50 µl aliquot of the supernatant was set aside for DNA cleanup and concentration measurement, and the rest aliquoted for future ChIP reactions. The DNA cleanup was performed by incubating the sample with 1 µl RNase A at 37°C for 15 minutes, then with 10 µl Proteinase K at 42°C for 1.5 hr. DNA was precipitated by phenol chloroform extraction 1:1 TE saturated pH 8 (Sigma, St Louis MO). The sample was centrifuged at maximum speed for 10 min at 4°C and the aqueous phase moved to a fresh tube. 20 µl of 3 M Sodium Acetate pH 5.2 and 500 µl 100% ethanol was added to the sample followed by overnight incubation at -80°C. The sample was then centrifuged for 15 minutes at 4°C, the pellet washed with 500 µl 70% ice cold ethanol, and resuspended with 30 µl double distilled ionized (DDI) water. Samples were quantified using a nanodrop and run on a 2% agarose gel at 100V for 45 min. The gel was

visualized using the Amersham Typhoon Imager Scanner FLA 9000 (GE Life Sciences, Piscataway NJ) with the optimal fragment size between 200-500 bp.

2.3 Chromatin Immunopercipitation and Validation

ChIP Reactions were set up in 100 µl volumes as described in the ChIP IT Express Kit (Active Motif) with minor modifications. The antibody concentration for H3K9Ac (Cat #39137 Active Motif) was used at a concentration of 5 µg instead of the recommended 1-3 µg. Sheared chromatin was used at a concentration of 30 µg. The samples were incubated overnight on a rotating platform, at 4°C. The rest of the protocol was followed without modification. DNA purification was performed using the Agencourt® Ampure XP DNA Purification beads (Cat #A63880 Beckman Coulter, Brea CA). Samples were fully resuspended with 200 µl of Ampure XP beads, and incubated for 10 min at room temperature. Using a magnetic stand the samples were washed with 200 µl of 70% ethanol. Beads were allowed to air dry on the magnet, and DNA eluted using nuclease free water. DNA was quantified using Nanodrop UV/VIS Spectrophotometer (Thermo Scientific, Wilmington DE). To ensure successful chromatin, pull downs were performed. RT-PCR was performed using commercially available positive and negative control primers. A positive primer for the promoter region of GAPDH was utilized (Cat # 71018 Active Motif), the negative control primers used were for a gene desert on chromosome 6 (Cat # 71011, Active Motif) and gene desert on chromosome 17 (Cat # 71012, Active Motif). RT-PCR was performed in 20 µl reaction volumes, utilizing 10 ng of DNA, 10ul SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 4

ul of the active motif primer. RT-PCR was performed using the ViiA7 Real-Time PCR System (Applied Biosystems) under the following conditions: 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Normalization of the data was performed using the ChIPed DNA of each sample against the inputs for each sample for both the positive and negative control primers using the $2^{-\Delta\Delta CT}$ method.

2.4 Library Preparation, Quality Check and Run Conditions

The next generation sequencing (NGS) library was prepared using the Ovation Ultralow System VS2 1-16 (Cat# 0344 Nugen San Carlos, CA). The samples were prepared at the Brown University Sequencing Facility, according to the manufactures protocols. Briefly, 25 ng of ChIPed DNA was end repaired, and adapters were added to each sample and ligated to distinguish samples from each other on the flow cell. Following ligation, the samples were purified and amplified by 10 cycles of PCR. Samples were quantified using the Qubit dsDNA BR Assay Kit (Cat # Q32850 Life Technologies, Carlsbad CA).

Fragment analysis was performed using the High Sensitivity NGS Fragment Analysis Kit (Cat# DNF-474 Advanced Analytical Technologies, Ankeny IA) using 2 µl of library input at a concentration of 4-6 ng/µl following the manufactures instructions. For each library 1:1000, 1:5000, and 1:1000 dilutions were made using the Kapa Library Quantification Kit (Cat #KK4873 Illumina San Diego, CA), each dilution was run in triplicate on ViiA7 Real-Time PCR System (Applied Biosystems). Reaction volume of 20 µl were run under the following conditions: 95°C for 5 min, then 35 cycles of 95°C for 20 sec, 60°C for 45 sec.

Samples were excluded from analysis if the standard deviation from the median was >0.1 C_t units. The total amount of library was determined for each library dilution using six know amplification standards with an amplification product size of 462 bp. The median concentration of the library was calculated using all three dilutions.

Samples were pooled and submitted for sequencing at 20 nM concentration to the Bauer Sequencing Facility at Harvard University. The samples were processed using the Illumina Hiseq 2500 system in rapid run mode producing 1 X 50 bp single reads. One sample was run on two lanes of a whole flow cell according to standard protocol performed at the Bauer center.

2.5 Bioinformatics Analysis

The raw data from ChiP-seq was analyzed in-house at the University of Rhode Island by the Biomedical Research Bioinformatics Core. Each tagged sample was end to end aligned to the mouse genome using Bowtie2 allowing for one mismatch between sequences [24, 25]. Files were then converted to bed files using SAMtools [26]. To determine H3K9Ac sites, peak calling was performed using SICER peak sites in the Pb group, as well as the control group. The Pb data set was then filtered against the control data set for each timepoint, leaving only peaks unique to the Pb exposed animal. The analysis was performed using a false discovery rate (FDR) of 0.01, and parameters specified from previously published protocols [27]. Peak annotation was later performed using the homer program with gene ontology and genome ontology [28].

3. Results

3.I H3K9Ac Promoter Analysis

The homer peak annotation output provided H3K9Ac peaks for all genomic regions including promoters, exons, introns and intergenic regions. For the purpose of this manuscript, these data were filtered out and the results reported here are only for promoter regions, defined by homer as -1000 to +1000 bp from the transcription start site (TSS). All comparisons were made using the filtered peak program from SICER, which subtracted control animal peaks at each timepoint from the Pb exposed animal peaks.

In all three data sets, peaks were sorted to corresponding genes to identify how many genes had increased H3K9Ac peaks in the promoter region. We identified 405 genes at day 20, 62 genes at day 270 and 7220 genes at day 700 with H3K9Ac binding peaks after subtracting the control group peaks from each data set (Figure 1). We then identified which genes were common between day 20 animals and day 700, as well as day 270 and day 700. For the day 20 samples, 293 genes had an increase in H3K9Ac peaks in their promoter, whereas only 112 genes were shared with the day 700. Similarly at day 270, 48 genes were unique, whereas 14 genes were shared with the day 700 group (Figure 2).

Data files were then run by Homer and analyzed against the Kyoto Encyclopedia of Genes and Genomes (KEGG). The KEGG pathway database has a specific pathway of genes implicated in AD (ko5010)[29, 30]. At the promoter level, Apbb1 was the only gene to have an increase in H3K9Ac peaks

at the promoter in day 20 mice. There were no genes found to have increased acetylation at the day 270 time-point. However, at day 700, 74 genes implicated in Alzheimer's were found to have increased H3K9Ac in the promoter region (Figure 3). A complete list of these genes can be found in Table 1.

For promoter regions, visualization of H3K9Ac islands are demonstrated in Figure 4. The degree of abundance is presented in the top trace in the UCSC browser[31]. The higher degree of shading indicates the higher abundance of reads to that specific sequence in the genome. Three genes of interest to Alzheimer's pathology are presented, Apoe, App and Mapt. In all three of the traces, there is a high degree of H3K9Ac binding in the region immediately before the first exon, and within the first exon and first intron (Figure 4). The acetylation peaks for a subset of the 74 genes were calculated (Figure 5). These gens were chosen based on their association with App and Mapt.

3.2 Genome Wide KEGG Analysis

This analysis utilized the entire genomic data set including all previously excluded elements (exons, introns, intergenic regions) to determine statistical significance. Using this technique, the filtered data identified a statistically significant increase in H3K9Ac peaks in the Alzheimer's pathway. At day 20, 29 genes were identified (P=0.0225) and at day 700, 96 genes were identified (P=.000103). A full list of these genes can be found in Table 2.

4. Discussion

ChiP sequencing is a powerful tool used to identify binding sites of transcription factors or histone marks to DNA regions. It is also the preferred

method when working with larger genomes such as the human or mouse genome[32]. The design and sequence run of this experiment was specifically chosen based on the number of reads required to detect significant changes in histone marks in the genome. The Encyclopedia of DNA Elements (ENCODE) consortia has identified the appropriate number of reads for mapping histone marks at a threshold greater than 10 million reads per sample [33]. This experiment yielded between 17-19 million reads per sample for H3K9Ac, a mark well known for its role in activating gene expression. Due to the abundance of genome reads, the analysis and figures were created to examine genes with increased acetylation sites in the promoter regions, rather than all genomic regions. Furthermore, a study by Roh et al., determined that H3K9Ac islands present in the promoter, as well as the 5'UTR, appear to be more involved in regulating gene expression than those acetylation sites present in all other regions of the genome [34].

These data indicate that there is an abundance of acetylation sites in a number of genes in animals developmentally exposed to Pb. Specifically, when we subtract the H3K9Ac peaks of the control animals at each time point from the Pb peaks we identified 7220 genes with distinct peaks at day 700, these effects were much smaller in the day 20, and day 270 timepoints (Figure 1). From our data, wee see there are considereably less peaks in the day 270 timepoint, which would suggest that consequences of developmental exposure to Pb manifest themselves at the end of life. Based on these data we would expect there to be an upregulation at the majority of the genes at day 700, due the role H3K9Ac in

regulating gene expression. For genes previously examined such as those involved in AD, this fits our previous findings [18, 23, 35]. However, these results will be validated in future studies examining genes in other pathways.

The KEGG genomic database links genes and genetic information to higher order functional categories, such as molecular function and disease pathology [29]. Specifically, the ko0510 pathway identified 162 genes that have been associated with AD pathology. These genes are involved in App processing, Tau phosphorylation, as well as mitochondrial and calcium dyfunction and endoplasmic reticulum stress. At day 700, 74 of these genes have an increase in acetylation peaks in their promoter regions (Table 1 and Figure 3). This increase in H3K9Ac peaks in the promoter indicates there is a high likelihood that these genes would in turn be upregulated following exposure to Pb. When we examined the entire genome using the KEGG database, we found statistically significant changes in a number of genes at both the day 20 timepoint, as well as the day 700 timepoint (Table 2). Although not the promoter region, these H3K9Ac could also be playing a role in regulating gene expression and will require further analysis.

Other studies have implicated histone acetylation involvement in memory impairment, as well as in specific gene loci related to AD [10, 36]. *In vitro* treatment with thyroid hormone lead to a decrease in H3K9Ac binding in the promoter of APP, and subsequently a decrease in expression, these effects were reduced with subsequent treatment with histone deacetylase inhibitors (HDAC)[37]. Similarly, in animal models of AD treatments with HDAC inhibitors

have been explored as potential therapeutics and have been shown to improve learning and memory deficits in animals [38]. While studies have reported hypoacetylation at genes related to memory and learning, others similar to our own have identified hyperacetylation of genes involved in the pathology [39]. The relationship between histone acetylation and AD is complex, and likely involves both cases of hypo as well as hyperacetylation of genes, as recently reviewed [36].

These data will be further analyzed using bioinformatics methods utilizing the entire genome, as well as specified promoter inputs. Although we focused our analysis on the promoter region in this manuscript due to the importance of acetylation sites in this region, there are known regulation sites throughout the genome further than 1000 bp from the TSS [40]. These enhancer elements have been shown by others to also interact with histone proteins to influence gene expression [40]. We will also analyze samples across time, independent of normalization to the control animals to identify ways in which Pb exposed mice differ from one another across their lifespan in acetylation peaks, the same analysis will be performed in the control group to analyze the effects of aging.

Although these findings are preliminary, they are novel in that we have identified a potential epigenetic mechanism by which these AD genes become upregulated in a model that we have worked with for over a decade. We have provided evidence for H3K9Ac as a potential regulator in a number of AD related gene promoters. This increase in H3K9Ac may contribute, or may be driving the increase in gene expression that we have seen in previous publications.

Acknowledgements

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Figure 1.

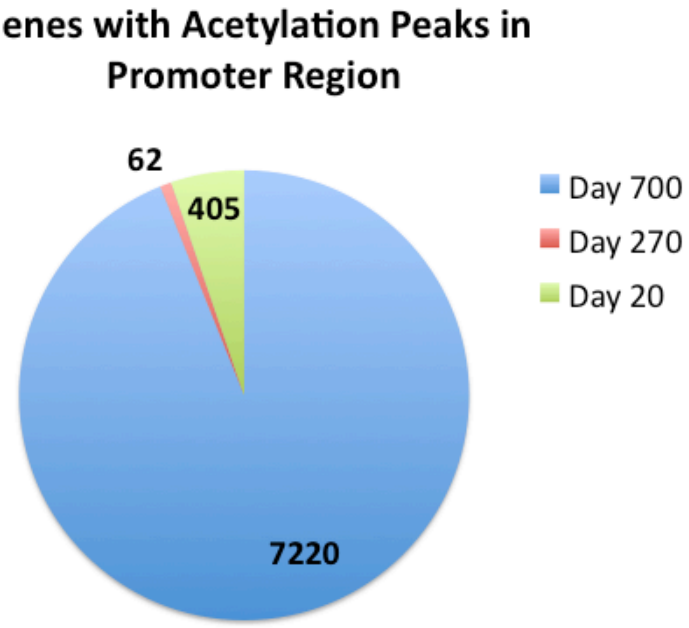

Figure 1. Acetylation Peaks in Promoter Regions. The total number of genes by time point (Blue); day 700 (red); day 270 and (green); day 20 Pb exposed animals normalized to their control counterparts with acetylated peaks in their promoter.

Figure 2.

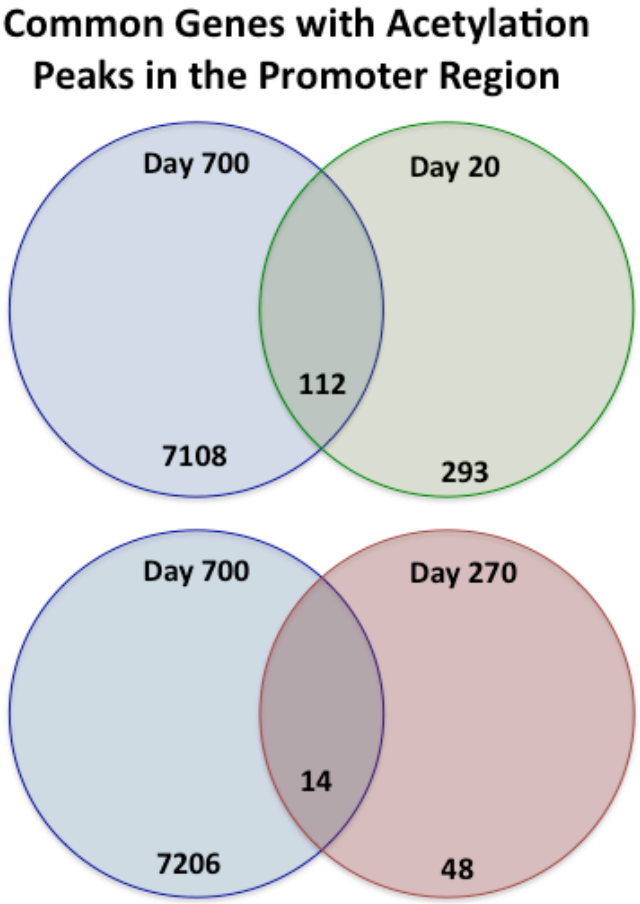

Figure 2. Common Genes with Acetylation Peaks in the Promoter Region. The total number of unique genes with acetylation peaks are shown for each time point in Pb exposed animals normalized to their control counterparts. The common number of genes shared between both time points is represented between them.

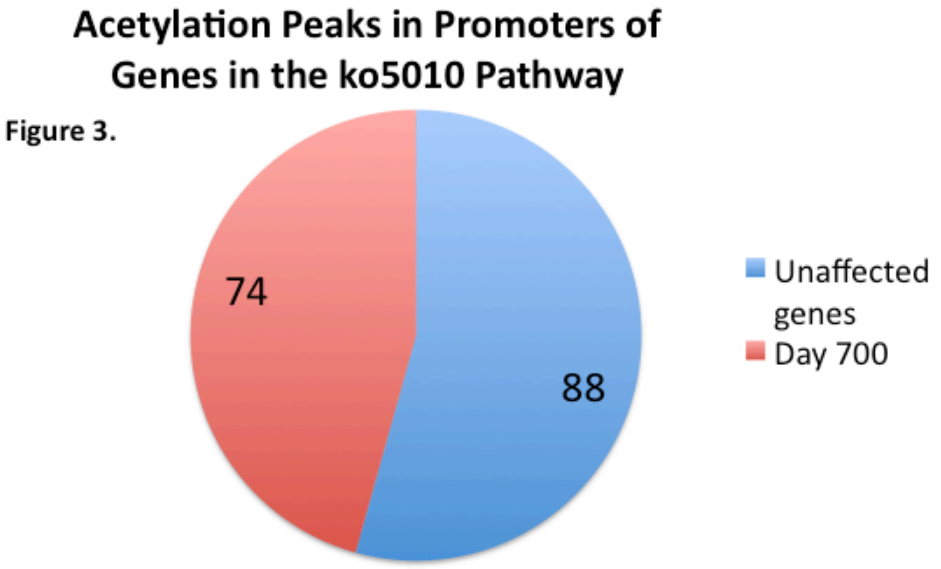

Figure 3. Acetylation Peaks in Promoters of Genes in the ko5010 Pathway. Of the 162 genes in the Alzheimer's k05010 pathway, 74 of them have genes with acetylated sites in their promoter in the Pb exposed animals at day 700 relative to their control counterparts.

Figure 4.

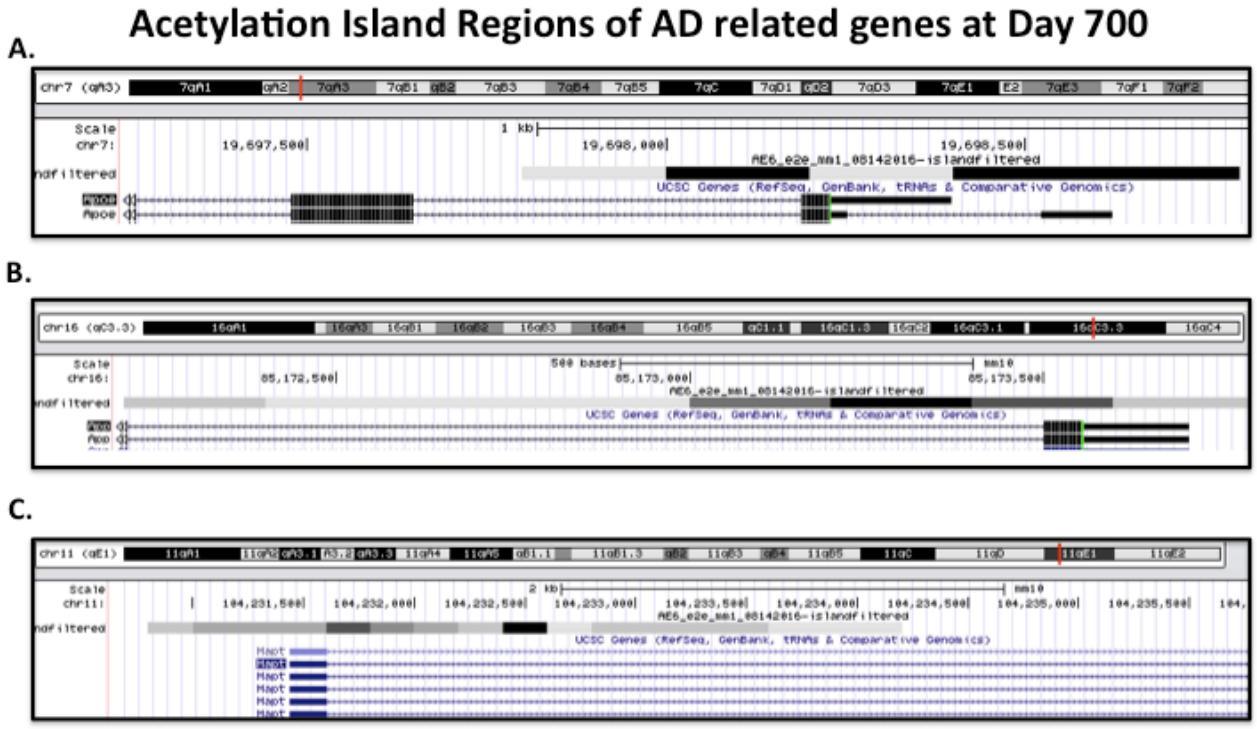

Figure 4. Acetylation Island Regions of AD related genes. The island filtered trace was created by filtering all of the control peaks from the Pb peaks at Day 700. This was then visualized using the UCSC genome web browser. The shading represents relative abundance of reads at that particular site or location, with the darkest shading indicating the most abundant reads in that region. The following genes are represented, A) Apoe, B) App, and C) Mapt, all indicating an enrichment of H3K9Ac either at or near their promoter regions.

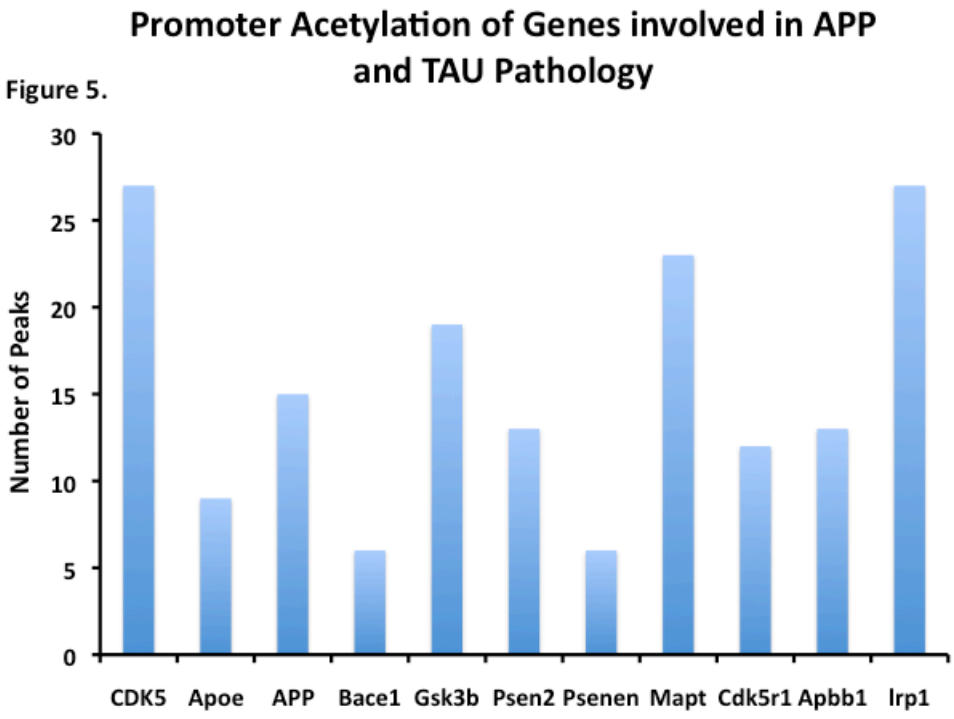

Figure 5. Promoter Acetylation of Genes involved in APP and Tau Pathology. Total number of acetylation sites were counted in the promoter region of genes involved in both APP and TAU pathology.

I. Pb day 700 normalized to Control day 700
Target genes: 74 **Tau regulation:** Cdk5, Mapt, Gsk3b, Cdk5r1 **APP processing:** Apoe, Psen2, Apbb1, Lrp1, Psenen, App, Bace1 **Mitochondrial, endoplasmic reticulum dysfunction and Ca2+ signaling:** Ndufa3, Ppp3cb, Ndufa8, Eif2ak3, Ndufc1, Ndufb7, Ppp3cc, Atp5g2, Sdhb, Plcb1, Adam10, Capn2, Apaf1, Atp5j, Itpr3, Atp5a1, Ndufa13, Ppp3ca, Ndufa4, Calm1, Cox5a, Gnaq, Cox5b, Ndufab1, Ndufb4, Cox6a1, Cycs, Ndufa6, Ern1, Grin2a, Grin2d, Calm2, Ndufa5, Gapdh, Casp9, Aph1a, Ppp3r1, Grin2c, Bad, Atp5f1, Calm3, Cox4i1, Aph1c, Ndufb2, Atp5d, Ndufa7, Ndufs1, Grin1, Cacna1c, Uqcrq, Capn1, Itpr2, Atp5g3, Ndufv1, Casp7, Atp5b, Mapk1, Ndufb6, Atp5h, Ncstn, Cyc1, Atp2a2, Uqcrfs1

Table 1. Presence of acetylation islands in promoters of genes associated with AD . The gene database used for analysis was the ko5010. Genes with acetylation islands in the Pb exposed group at day 700, which were absent in the day 700 control group.

A. Pb day 20 normalized to Control day 20
Target genes: 29 P value: 0.022533815
Tau regulation: Mapt, Cdk5r1
APP processing: Apbb1, Psen2, Lrp1, Ide
Mitochondrial, endoplasmic reticulum dysfunction and Ca2+ signaling: Cacna1c, Capn1, Atp2a3, Atp5o, Ndufs7, Ndufs8, Ndufb7, Uqcr10, Cox7a1, Uqcr11, Capn2, Cacna1d, Ndufb9, Nos1, Grin2d, Calm2, Cox7a2l, Cacna1s, Atp2a2, Cox8b, Ndufa4l2, Grin2c, Ndufa13
B. Pb day 700 normalized to Control day 700
Target genes: 96 P value: 0.000103327
Tau regulation: Mapt, Cdk5, Cdk5r1, Gsk3b
APP processing: Apoe, Psen2, Bace2, Psenen, Bace1, App, Lrp1, Apbb1
Mitochondrial, endoplasmic reticulum dysfunction and Ca2+ signaling: Ndufa3, Ndufs7, Ppp3cb, Ndufa8, Eif2ak3, Ndufc1, Ndufb7, Ppp3cc, Atp5g2, Sdhb, Plcb1, Adam10, Cacna1d, Capn2, Cox8c, Apaf1, Atp5j, Itpr3, Atp5a1, Ndufa4l2, Ndufa13, Ppp3ca, Ndufa4, Calm1, Cox5a, Ryr3, Cox6a1, Cycs, Ndufa6, Ern1, Grin2a, Nos1, Grin2d, Calm2, Ide, Ndufa5, , Uqcrfs1, Cox7c, Atf6, Ppp3r1, Grin2c, Cox6b2, Bad, Atp5f1, Calm3, Cox4i1, Aph1c, Ndufb2, Atp5d, Uqcrb, Calm5, Ndufa7, Grin2b, Ndufs1, Grin1, Gnaq, Cox5b, Ndufab1, Plcb3, Ndufb4, Atp5o, Cacna1c, Uqcrq, Ndufa12, Capn1, Itpr2, Atp5g3, Ndufv1, Casp7, Atp5b, Mapk1, Ndufb6, Atp5h, Ncstn, Cacna1s, Ndufs4, Cyc1, Plcb4, Atp2a2, Casp8, Gapdh, Casp9, Aph1a, Mapk3

Table 2. Presence of acetylation islands in genes associated in AD as defined by the KEGG ko5010 database. (A) List of genes with statistically significant ($p < 0.05$) acetylation islands in the Pb exposed group at day 20, which were absent in the control day 20. (B). List of genes with statistically significant ($p < 0.001$) acetylation islands in the Pb exposed group at day 700, which were absent in the day 700 control.

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Appendix 1
A Mechanistic Proposal for The Actions of Lead on the Aging Brain

In our model of developmental lead (Pb) exposure, male C57BL6J pups are exposed to 0.2% Pb acetate through the drinking water of the dam, and the control group receives regular tap water from postnatal day (PND) 1-20. Previous publications by us have shown that this level of Pb results in a concentration in the cerebellum of PND 20 rodents (0.25 +/- 0.07 µg/g [1], and blood lead level has been shown to be 46.43 µg/dl during Pb exposure, both measurements are undetectable in adults [2]. As amply cited in the manuscripts of this dissertation, we have published that this developmental exposure is accompanied by a transient increase during development and a latent increase in Alzheimer's disease (AD) related biomarkers in old age [3-5]. We have also previously reported that exposure to Pb during early life results in global hypermethylation patterns as well as global downregulation of many genes [6, 7]. However, a small subset of genes exhibit either hypomethylation or are up-regulated by pathways independent of DNA methylation; AD-related genes are among them.

In manuscript III, we have reported a number of genes involved in AD with increased acetylation peaks in their promoter region. Histone acetylation at gene promoters is indicative of increased gene expression, and would support our model and previous findings. From these new data uncovered by our ChIP-seq study with histone acetylation, we hypothesize the following mechanisms of action by which Pb may be acting to modify gene expression.

Our ChiP-seq data indicates that a number of histone deacetylase (HDAC) genes lack histone acetylation peaks in their promoters, both in early and late in

life. Therefore we would expect the expression of these genes to be down-regulated. This may be occurring by DNA methylation patterns that are more stable and persist over time, especially in neurons where we have little to no turnover in cells. Therefore, our Pb exposure may be enhancing DNA methylation at these gene promoters. HDAC proteins function primarily to deacetylate histones, if we are repressing these proteins we could then in turn be enhancing histone acetylation throughout the lifespan, which is what is observed in our data set.

We also believe that HDAC proteins may be directly targeted during the exposure period in early life due to the fact that HDAC proteins have a zinc finger-binding domain. Early work by the Zawia lab in the 1990s has shown that Pb can interact and disrupt zinc finger proteins, making HDAC proteins a prime target. This maybe one mechanism by which Pb is modifying gene expression early in life, it may also partially explain our global reprogramming of genes both by histone proteins as well as DNA methylation.

The changes observed in our animal model are greatly exacerbated in the aging brain, and we believe this is due to an increase in a number of different pathways such as, an increase in reactive oxygen species, increased inflammatory responses, and mitochondrial dysfunction. We believe that early life reprogramming of gene expression due to environmental exposure undermine our ability to combat insults that occurring during aging.

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